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The Role of CaMKII Delta in Pathophysiological Responses Induced by Beta-Adrenergic Receptor Stimulation and Angiotensin II

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

in

Biomedical Sciences

by

Andrew Willeford

Committee in charge:

Professor Joan Heller Brown, Chair
Professor Barry Greenberg
Professor Åsa Gustafsson
Professor Tracy Handel
Professor Paul Insel
Professor Andrew McCulloch

2017

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University of California, San Diego

2017

TABLE OF CONTENTS

Signature Page iii

Table of Contents iv

List of Figures vii

Acknowledgments x

Vita xi

Abstract of the Dissertation xii

Chapter 1 1
1.1 CaMKIIδ Signaling 1
1.2 CaMKIIδ in cardiovascular disease 3
1.3 CaMKIIδ in inflammation 6
1.4 β-adrenergic receptor stimulation, angiotensin II, and CaMKIIδ 8
1.5 Conclusion 9

Chapter 2 12
2.1 Summary 12
2.2 Introduction 13
2.3 Results 16
2.3.1 Characterization of CaMKIIδ-KO mice 16
2.3.2 Deletion of CaMKIIδ reduces phosphorylation of Ca^{2+} handling proteins in adult cardiac myocytes 17
2.3.3 Deletion of CaMKIIδ reduces diastolic SR Ca^{2+} leak 19
2.3.4 Fight-or-flight response to acute β-AR stimulation is not altered in CaMKIIδ KO mice 22
2.3.5 Deletion of CaMKIIδ has no effect on cardiac hypertrophy ... 23
2.3.6 CaMKII deletion inhibits fibrosis induced by chronic β-AR stimulation 27
2.3.7 CaMKII deletion protects from heart failure development induced by sustained β-AR stimulation 29
2.3.7 Inactivation of the CaMKII phosphorylation site RyR2 inhibits development of heart failure induced by chronic β-AR stimulation 31
2.4 Discussion 32
2.5 Experimental Procedures 39
2.5.1 Gene array and quantitative PCR 39
2.5.2 Isolation of adult mouse ventricular myocytes 40
2.5.3 Line Scan Confocal Microscopy 41
2.5.4 CaMKII activity assay 42
2.5.5 Isolated Heart Studies 42

2.5.6 Histological analysis 43
2.5.7 Immunoblotting 44
2.5.8 Isoproterenol injection and osmotic pump delivery 44
2.5.9 Transthoracic echocardiography 45
2.5.10 Statistics 46
2.6 Acknowledgements 46

Chapter 3 48
3.1 Summary 48
3.2 Introduction 50
3.3 Results 53
3.3.1 Angiotensin II infusion activates CaMKIIδ and NFkB 53
3.3.2 CaMKIIδ activation in cardiomyocytes mediates angiotensin II-induced cardiac inflammatory chemokine and cytokine expression 55
3.3.3 Angiotensin II-induced early inflammatory responses are not secondary to cell death or hypertension 58
3.3.4 Angiotensin II recruits macrophages to the heart through CaMKIIδ and MCP-1 60
3.3.5 Angiotensin II activates the NLRP3 inflammasome through CaMKIIδ 62
3.3.6 Active CaMKIIδ regulates NLRP3 inflammasome priming and ROS-dependent inflammasome activation in cardiomyocytes 65
3.3.7 Inhibition of the inflammasome attenuates Ang II-induced inflammation 66
3.3.8 Cardiomyocyte CaMKIIδ signaling contributes to angiotensin II-induced fibrosis 67
3.3.9 MCP-1 and the inflammasome are involved in angiotensin II-induced fibrosis 69
3.4 Discussion 72
3.5 Experimental Procedures 77
3.5.1 Animals 77
3.5.2 CaMKII activity assay 78
3.5.3 Nuclear fractionation 79
3.5.4 Adult mouse ventricular myocyte isolation 79
3.5.5 Histological Analyses 80
3.5.6 Cell Death 81
3.5.7 RNA extraction, cDNA synthesis, and quantitative PCR 81
3.5.8 Caspase 1 activity assay 81
3.5.9 Neonatal rat ventricular myocyte isolation, culture, and infection 82
3.5.10 Immunoblotting 83

3.5.11 Statistical analyses 83
3.6 Acknowledgements 84
Chapter 4 85
4.1 Summary 85
4.2 Future Studies 88
4.2.1 Cellular location of inflammasome signaling in Ang II-induced inflammatory responses and contribution of interleukin 1β 88
4.2.2 Differential pro-inflammatory signaling by CaMKIIδ splice variants 89
4.2.3 Contribution of monocyte chemotactic protein-1 expression in cardiomyocytes to Ang II-induced inflammatory responses 90
4.2.4 Role of CaMKIIδ-mediated inflammatory signaling in development of adverse remodeling and dysfunction in heart failure 91
4.3 Concluding Remarks 91
Appendix 93
Bibliography 101

LIST OF FIGURES

Figure 2.1. CaMK isoform expression and activity in CaMKIIδ-KO mice . 17

Figure 2.2. Effect of CaMKIIδ deletion on the phosphorylation of CaMKII targets in response to acute β-AR stimulation 19

Figure 2.3. CaMKIIδ is critical for ISO-induced SR Ca^{2+} leak 21

Figure 2.4. Effects of acute ISO exposure (1 μmol/L) on CaMKII activity, contractility and spontaneous beating rate of isovolumic Langendorff perfused hearts 23

Figure 2.5. CaMKIIδ deletion does not inhibit cardiac hypertrophy in response to chronic β-AR stimulation 25

Figure 2.6. Chronic infusion of ISO does not increase the phosphorylation of Akt 26

Figure 2.7. CaMKIIδ deletion inhibits cardiac fibrosis after 2-week continuous ISO infusion..... 28

Figure 2.8. Cardiomyocyte-specific CaMKIIδ deletion attenuates cardiac fibrosis induced by 4 weeks ISO injection..... 29

Figure 2.10. Echocardiographic parameters of wild-type and S2814A mice at one (1-wk) and four (4-wk) weeks of chronic ISO infusion 32

Figure 3.1. Angiotensin II infusion activates CaMKIIδ and elicits NFkB signaling through CaMKIIδ 54

Figure 3.2. Deletion of CaMKIIδ in the cardiomyocyte attenuates Ang II-induced inflammatory gene expression in the heart and monocyte chemotactic protein 1 expression in the myocyte..... 56

Figure 3.4. Angiotensin II infusion for 1 day does not cause cardiac cell death..... 59

Figure 3.5. Angiotensin II infusion increases blood pressure to similar extents in control and cardiomyocyte-specific CaMKIIδ KO mice..... 60

Figure 3.6. Ang II-induced macrophage accumulation in the heart is attenuated by CaMKIIδ gene deletion in the cardiomyocyte and MCP-1 receptor blockade 62

Figure 3.7. Ang II-induced priming and activation of the inflammasome is mediated through cardiomyocyte CaMKIIδ and NFkB..... 64

Figure 3.8. CaMKIIδ primes and activates the inflammasome in neonatal rat ventricular myocytes (NRVMs)..... 66

Figure 3.9. Inflammasome inhibition attenuates Ang II-induced inflammatory responses in the heart 67

Figure 3.10. Cardiac fibrosis induced by 7 day Ang II infusion is attenuated in mice lacking CaMKIIδ in the cardiomyocyte 68

Figure 3.11. Angiotensin II induces profibrotic mediator expression through CaMKIIδ signaling in the cardiomyocyte..... 69

Figure 3.12. Inhibition of MCP-1 or inflammasome signaling attenuates cardiac fibrosis induced by Ang II infusion 71

Figure 4.1. Proposed pathway by which cardiomyocyte CaMKIIδ initiates Ang II induced inflammation and fibrosis 87

Figure A.1. General structure of the multimeric holoenzyme CaMKII..... 93

Figure A.2. Mechanism of CaMKII activation by post-translational modification (PTM)..... 93

Figure A.3. Hypothesis of CaMKIIδ-mediated inflammation in response to a non-ischemic stress i.e. angiotensin II..... 94

Figure A.4. Isoproterenol (ISO) infusion for 1 week induces monocyte chemotactic protein 1 (MCP-1) expression in the heart and requires CaMKIIδ 94

Figure A.5. Ang II activates CaMKIIδ in the isolated perfused heart..... 94

Figure A.6. Ang II induces CaMKIIδ-dependent inflammatory gene expression in isolated adult mouse ventricular cardiomyocytes *in vitro* 95

Figure A.7. Inflammatory gene expression induced by Ang II *in vitro* requires nuclear factor kappa B 95

Figure A.8. Cardiac hypertrophy induced by 7 day Ang II infusion does not require CaMKIIδ 96

Figure A.9. Cardiomyocyte CCN1 deletion does not protect the heart from Ang II-induced fibrosis 97

Figure A.10. Transgenic overexpression of the δ_C but not of the δ_B splice variant increases gene expression of multiple pro-inflammatory chemokines and cytokines..... 97

Figure A.11. Inflammatory cytokine and chemokine gene expression in the heart is not induced by electronic cigarette vapor exposure 99

Figure A.12. Fibrotic gene expression is moderately increased in whole hearts of mice exposed to electronic cigarette vapor for 1-3 months 100

Figure A.13. Chronic nicotine infusion does not alter inflammatory gene expression induced in the heart by intraperitoneal LPS injection 100

Figure A.14. TNFa release stimulated by *in* vitro LPS treatment in peritoneal macrophages is inhibited by nicotine but not altered in macrophages isolated from mice infused with nicotine 100

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Chapter 3, in full, is currently being prepared for submission for publication upon completion of follow-up studies. The dissertation author is the primary author in collaboration with Takeshi Suetomi, Audrey Nickle, Shigeki Miyamoto, and Joan Heller Brown.

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ABSTRACT OF THE DISSERTATION

The Role of CaMKIIδ in Pathophysiological Responses Induced by Beta-Adrenergic Receptor Stimulation and Angiotensin II

by

Andrew Willeford

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2017

Professor Joan Heller Brown, Chair

The initial studies of my graduate research, which resulted in a co-authorship and for clarity reasons is presented in this dissertation in full, utilized calcium/calmodulin-dependent protein kinase II δ (CaMKIIδ) global knockout mice to determine the requirement for the kinase in cardiac remodeling and heart failure induced by chronic β-adrenergic receptor (β-AR) stimulation. Specifically, my work focused on the requirement of CaMKIIδ in hypertrophic and fibrotic responses induced

by chronic treatment with the β-adrenergic receptor agonist isoproterenol (ISO). We found that hypertrophy induced by daily ISO injection or infusion for up to 4 weeks was preserved in animals lacking CaMKIIδ. In contrast, fibrotic responses induced by the same dosing regimen were attenuated by CaMKIIδ gene deletion.

The primary work in my dissertation utilized a cardiomyocyte-specific CaMKIIδ knockout mouse model to study the role of cardiomyocyte CaMKIIδ in inflammatory and fibrotic responses induced by angiotensin III (Ang II). We hypothesized that CaMKIIδ initiates inflammatory gene expression and inflammatory cell recruitment and leads to cardiac fibrosis. Knockout mice in which CaMKIIδ is specifically deleted in cardiomyocytes showed attenuated inflammatory gene expression induced by 1-day Ang II infusion as compared to control ($CaMKII\delta^{fl/fl}$) mice. These responses were dissociated from cell death and differences in blood pressure. Monocyte chemotactic protein 1 (MCP-1) was induced in cardiomyocytes in response to Ang II and this response was found to be dependent on CaMKIIδ. Macrophage recruitment induced by 1-day Ang II infusion was also found to be dependent on CaMKIIδ signaling in the cardiomyocyte. Ang II infusion primed and activated the inflammasome and expression of CaMKIIδ in cardiomyocytes *in vitro* also primed and activated the inflammasome

through a reactive oxygen species-dependent pathway. Pharmacological inhibition of either the receptor to MCP-1 or the inflammasome attenuated Ang II-induced inflammatory responses. Longer term Ang II infusion elicited robust fibrosis and this response was attenuated in mice lacking CaMKIIδ in the cardiomyocyte or treated with either inhibitor. These results demonstrate a critical role for a cardiomyocyte autonomous transcriptional pathway, triggered by CaMKIIδ in the cardiomyocyte, in inflammatory responses induced by Ang II and leading to fibrosis.

Chapter 1

Introduction

1.1 CaMKIIδ Signaling

The calcium and calmodulin-dependent protein kinase II (CaMKII) is a holoenzyme that exists as a dimer of two hexameric rings[1] (Fig. A.1). There are four known CaMKII isoforms (α, β, γ, and δ), all of which have different tissue distribution. CaMKIIα and β are expressed primarily in the brain while CaMKIIγ and δ have a wider tissue distribution[2, 3]. The CaMKIIδ isoform is the isoform of interest in this work because it is the predominate form in the myocardium. CaMKIIδ exists as multiple splice variants, the major two being CaMKIIδ$_B$ and CaMKIIδ$_C$[4]. Each isoform is under intense study but current understanding of their specific roles is incomplete.

CaMKIIδ phosphorylates various substrates including ion channels and calcium handling proteins in the heart, causing electrophysiological changes that can lead to arrhythmogenesis and contractile dysfunction[5, 6]. Other less well studied targets of CaMKIIδ include proteins involved in gene transcription. CaMKIIδ has been demonstrated to play a role in activation of the cardiomyocyte fetal gene program through its effect on class II histone deacetylases. Phosphorylation of HDACs by CaMKIIδ inhibits HDAC-mediated repression of MEF2, allowing MEF2-dependent gene expression[7-9]. CaMKIIδ has also been shown to play a role in activating the master regulator of inflammatory gene expression, nuclear factor kappa B (NFkB)[10-12]. Other pro-inflammatory transcriptional regulators linked to CaMKIIδ include activator protein 1 (AP-1) and signal transducer and activator of transcription 3 (STAT3)[13-16]. The mechanisms CaMKIIδ uses to regulate these transcription factors and the downstream effects of their activation has received only limited attention.

Each of the 12 subunits of CaMKII contains an N-terminal catalytic domain, regulatory domain, and C-terminal association domain[1]. At baseline, the catalytic domain of CaMKII is constrained by the regulatory domain. Upon binding of calcium-liganded calmodulin (Ca/CaM) to the regulatory domain, CaMKII is structurally altered enabling its catalytic

activity and autophosphorylation[1]. Autophosphorylation confers Ca/CaM-independent activity such that the enzyme remains active after Ca/CaM dissociation. Other post-translational modifications including oxidation, nitrosylation, and O-GlcNAcylation of specific residues within the regulatory domain also exist and confer Ca/CaM-independent activity[17-19] (Fig. A.2).

1.2 CaMKIIδ in cardiovascular disease

Sustained activation of CaMKIIδ has important effects on the pathogenesis and progression of cardiovascular disease. Pressure overload mediated by transverse aortic constriction (TAC), myocardial infarction (MI), and ischemia/reperfusion injury (I/R) have been demonstrated to induce cardiac injury through CaMKIIδ signaling in studies using knockout and genetically altered mice[11, 20-22]. Furthermore, CaMKIIδ expression and activity is increased in the myocardium of heart failure patients[23, 24], a possible result of dysregulated calcium handling and increased neurohumoral signaling in the heart. CaMKIIδ is activated following stimulation of various G-protein coupled receptors involved in cardiovascular disease such as the β-adrenergic, endothelin 1, and angiotensin II receptors[19, 25, 26]. The downstream effects mediated by CaMKIIδ following stimulation of these receptors have been linked to altered calcium handling. Whether CaMKIIδ mediates effects through

the triggering of a transcriptional program has not been fully appreciated.

Cardiomyocyte cell death is a central component of heart failure development[27, 28] and CaMKIIδ has been demonstrated to be a major driver of cell death[29-33]. Ischemic injury induced by MI and I/R depletes myocytes of nutrition, induces ROS generation, and results in massive cell death[34]. Apoptosis induced by MI and acute high dose isoproterenol administration was shown to be attenuated in studies using transgenic expression of a CaMKII-specific inhibitory peptide (AC3-I)[31]. Furthermore, studies from our laboratory demonstrated that genetic deletion of CaMKIIδ significantly attenuates apoptosis induced by long-term pressure overload and ischemia/reperfusion[11, 22].

Published studies from our laboratory using CaMKIIδ knockout mice also show a requirement for CaMKIIδ in fibrosis induced by TAC, Gαq overexpression, and sustained β-adrenergic receptor stimulation[22, 35, 36]. Other studies have shown that fibrosis induced by MI and I/R is attenuated when CaMKIIδ is inhibited[20, 37]. Fibrosis occurs as a means of replacing heart tissue following cardiomyocyte cell death but is considered to be a downstream consequence of inflammation[38-41]. While it is possible that CaMKIIδ mediates fibrosis through its ability to increase cell death, we propose CaMKIIδ-mediated inflammation as a

major mechanism by which it contributes to fibrosis, as discussed further in section 1.3.

The two major splice variants of CaMKIIδ in the heart, which differ only by the inclusion of an 11-amino acid nuclear localization sequence in CaMKIIδ$_B$[4], appear to have different roles in cardiac pathophysiology. CaMKIIδ$_C$ is implicated in cell death in response to various stimuli and has been suggested to drive cell death through several mechanisms including upregulation of p53[42, 43]. In contrast, CaMKIIδ$_B$ appears to have a protective effect with studies suggesting that CaMKIIδ$_B$ mediates positive effects on Bcl-2 and heat shock factor 1[44, 45]. This pro-injury versus pro-survival duality between the splice variants is recapitulated in studies from our laboratory which utilize cardiomyocyte specific transgenic expression of CaMKIIδ$_B$ or CaMKIIδ$_C$. Mice overexpressing CaMKIIδ$_C$ spontaneously progress to hypertrophy and heart failure[46] while mice expressing CaMKIIδ$_B$ progress to hypertrophy but do not undergo cardiac decompensation[47]. Recent studies from our laboratory also indicate contrasting roles in activating pro-inflammatory pathways following I/R[10]. This distinction between CaMKIIδ$_B$ and CaMKIIδ$_C$ may explain in part why we observe pronounced differences in the transgenic mouse models expressing CaMKIIδ$_B$ vs CaMKIIδ$_C$ splice variants.

1.3 CaMKIIδ in inflammation

Inflammation is gaining growing attention as a key player in heart failure. There are elevated serum concentrations of multiple inflammatory cytokines in heart failure patients[48-50] and this is thought to contribute to myocardial fibrosis, dysfunction, and myocyte apoptosis[51]. Inflammation has been implicated in the pathogenesis of cardiac fibrosis, a key characteristic of heart failure[38-41]. How inflammation can drive cardiac fibrosis and decompensation remains unclear. Thus, exploring the pathways leading to cardiac inflammation and their role in fibrosis may provide insights to developing novel therapeutics for heart failure and other cardiovascular diseases.

Initiation of cardiac inflammation following ischemic injury (e.g. MI or I/R) has been linked to release of damage-associated molecular patterns (DAMPS) from necrotic cells in the heart[52, 53]. DAMPS act upon toll like receptors to initiate pro-inflammatory transcriptional pathways that induce expression and release of pro-inflammatory cytokines and chemokines[54-60]. How inflammation is initiated in cases of non-ischemic injury where cell death is not yet present remains unclear. Ang II infusion and pressure overload, two non-ischemic injury models, have been demonstrated to induce cardiac inflammation at early time points at which these interventions are not typically considered to induce cell

death[38, 61-71]. We hypothesize that this inflammatory response is initiated through CaMKIIδ and NFkB (Fig. A.3).

CaMKIIδ has been suggested to lead to inflammation induced by ischemic insult such as MI and I/R but only limited endpoints indicative of inflammatory changes have been examined. In one study using mice expressing a CaMKIIδ-specific peptide inhibitor (AC3-I), CaMKIIδ activated during MI was demonstrated to regulate the expression of the immune system enhancer, complement factor b[12]. Another study showed that CaMKIIδ is activated downstream of the RIP3 kinase pathway and that this pathway mediates expression of the pro-inflammatory genes interleukin 6 (IL-6) and tumor necrosis factor a (TNFa)[32]. A study from the Backs laboratory demonstrated that deletion of both CaMKIIδ and CaMKIIγ attenuates inflammatory cell accumulation in the heart following I/R and that the chemokine monocyte inflammatory protein 1 alpha can be upregulated and secreted from the cardiomyocyte in response to hypoxia[37]. None of these studies have determined a mechanism by which CaMKIIδ activated by ischemic insult leads to inflammatory responses. However, studies from our laboratory demonstrated that CaMKIIδ deletion attenuates NFkB activation induced by I/R and that this response is initiated in the cardiomyocyte[11]. In more recent studies, we showed that

the CaMKIIδ$_C$ isoform selectively activates NFkB and upregulates expression of IL-6 and TNFa following *ex vivo* I/R[10]. Although we have shown that CaMKIIδ activates NFkB in ischemic injury where cell death is present and likely potentiated by CaMKIIδ signaling, we hypothesize that CaMKIIδ could activate NFkB in the absence of cell death.

1.4 β-adrenergic receptor stimulation, angiotensin II, and CaMKIIδ

In heart failure, the body responds to a loss in cardiac contractile power by increasing the activity of the sympathetic system, effectively increasing β-adrenergic receptor stimulation of the heart. This increased β-adrenergic drive is a major player in the propagation of heart failure and is a primary target for therapy in heart failure patients[72]. β-adrenergic receptor stimulation is known to activate CaMKIIδ through proposed pathways involving canonical effects of PKA on calcium channels[73], Epac and PKC activation[73-76], β-arrestin[77], or nitric oxide[17, 78]. Published studies, described in chapter 2, show that CaMKIIδ mediates cardiac fibrosis and decompensation in response to sustained isoproterenol (ISO) treatment. Our experiments suggested that heart failure development in this model is mediated through CaMKIIδ-dependent phosphorylation of the ryanodine receptor[35]. Unpublished data from our laboratory also show that ISO induces monocyte

chemotactic protein 1 gene expression in the heart through CaMKIIδ (Fig. A.4). Thus, our work with CaMKIIδ in sustained β-adrenergic receptor stimulation led us to ask whether CaMKIIδ mediates cardiac decompensation not only through phosphorylation of the ryanodine receptor but also through initiation of inflammation.

To better study the extent to which CaMKIIδ may mediate cardiac inflammation in cardiac disease, we utilized chronic Ang II infusion. Angiotensin II plays a central role in nearly all cardiovascular diseases and is a primary target in the treatment of heart failure[79]. Chronic Ang II infusion is a well-established, non-ischemic cardiac inflammation model that elicits robust pro-inflammatory gene induction, leukocyte recruitment, and fibrosis[38, 64, 66, 70, 80]. The site at which Ang II initiates inflammation is typically considered to be in the endothelium and other non-myocytes[61]. Ang II receptors are present on cardiomyocytes[81-83] and Ang II can activate CaMKIIδ[19, 84, 85]. However, whether the cardiomyocyte or CaMKIIδ play a role in Ang II-induced cardiac inflammation has not been considered.

1.5 Conclusion

Recent studies have indicated a strong linkage between CaMKIIδ and cardiac inflammation. The work presented here strengthens that linkage and elucidates novel pro-inflammatory signaling pathways to

which CaMKIIδ within the cardiomyocyte compartment can initiate cardiac inflammatory gene expression and inflammatory cell recruitment without acute cell death. While inflammation has been associated with heart failure, clinical trials using inhibitors of inflammatory factors in heart failure therapy have failed to significantly improve patient survival[48, 49]. The failure of these trials may be explained by an inability of an inhibitor to stop a cascade of humoral factors already set in motion. Thus, understanding the initiating events of cardiac inflammation and inhibiting their occurrence, as might be achieved through CaMKIIδ inhibition, could provide significant benefit in the field of heart failure therapy.

Chapter 2, the work on the role of CaMKIIδ in β-adrenergic responses, was the project I contributed to when I first joined the laboratory. I have included the entire paper to allow the study to be presented clearly. My direct contributions investigated the effect of CaMKIIδ deletion on fibrotic and hypertrophic responses to chronic β-adrenergic receptor stimulation and include data presented in figures 2.5E and F, 2.6, and 2.9D.

Chapter 3 (and data in the appendix) in its entirety represents the primary results of my thesis research, including numerous studies that I

designed, interpreted, and executed. This is the portion of the thesis of which I am most proud, and for which I take full ownership.

Chapter 2

CaMKIIδ mediates β-adrenergic effects on RyR2 phosphorylation and SR Ca^{2+} leak and the pathophysiological response to chronic β-adrenergic stimulation

2.1 Summary

Chronic activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been implicated in the deleterious effects of β-adrenergic receptor (β-AR) signaling on the heart, in part by enhancing RyR2-mediated sarcoplasmic reticulum (SR) Ca^{2+} leak. We used CaMKIIδ knockout (CaMKIIδ-KO) mice, and knock-in mice with an inactivated CaMKII site S2814 on the ryanodine receptor type-2 (S2814A), to interrogate the involvement of these processes in β-AR signaling and

cardiac remodeling. Langendorff-perfused hearts from CaMKIIδ-KO mice showed inotropic and chronotropic responses to isoproterenol (ISO) that were similar to those of wild type (WT) mice, however in CaMKIIδ-KO mice CaMKII phosphorylation of phospholamban and RyR2 was decreased and isolated myocytes from CaMKIIδ-KO mice had reduced SR Ca^{2+} leak in response to isoproterenol (ISO). Chronic catecholamine stress with ISO induced comparable increases in relative heart weight and other measures of hypertrophy from day 9 through week 4 in WT and CaMKIIδ-KO mice, but development of cardiac fibrosis was prevented in CaMKIIδ-KO animals. A 4-week challenge with ISO resulted in reduced cardiac function and pulmonary congestion in WT, but not in CaMKIIδ-KO or S2814A mice, implicating CaMKIIδ-dependent phosphorylation of RyR2-S2814 in the cardiomyopathy, independent of hypertrophy, induced by prolonged β-AR stimulation.

2.2 Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional signaling molecule that has been implicated in pathological cardiac remodeling in response to stress[86]. 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[18, 19, 87]. In

response to activation of G-protein coupled receptors (GPCRs) by agonists such as endothelin or phenylephrine, CaMKIIδ is phosphorylated at Thr287, possibly resulting from release of Ca^{2+} from $InsP_3$-sensitive stores[26]. Other GPCR agonists such as angiotensin, that are known to induce oxidative stress, lead to CaMKII activation via oxidation of Met281/282[19]. There is growing evidence that β-AR stimulation can also activate CaMKII, although the signaling mechanisms through which this occurs remain controversial[88, 89]. Proposed mechanisms include activation of CaMKII through cAMP and PKA effects on Ca^{2+} channels[73], through Epac and PKC activation[74-76], through β-arrestin[77], or by a cAMP-independent mechanism which appears to involve nitric oxide[17, 78].

A relationship between β-adrenergic stimulation and CaMKII activity, especially in more chronic settings, is intriguing because both β-adrenergic and CaMKII pathways have been independently implicated in the development of heart failure[22, 72, 86, 88, 90]. Sustained β-AR stimulation resulting from increased sympathetic tone underlies the efficacy of β-AR blockers in heart failure treatment[72]. While CaMKIIδ has not yet been therapeutically targeted in patients, increases in CaMKIIδ expression and activation have been widely observed in animal and human models of heart failure[23, 46, 91, 92]. Inhibition of CaMKIIδ attenuates cardiac dysfunction and remodeling in mice subjected to sustained β-AR

stimulation[90]. Furthermore, inhibition of CaMKIIδ in human myocytes from patients with heart failure increases their contractile function[93]. Thus, CaMKIIδ and its targets may represent major downstream signals for the pathological remodeling induced by chronic β-adrenergic stress.

The studies reported here utilize CaMKIIδ knockout (CaMKIIδ-KO) mice to specifically determine the role of CaMKIIδ activity in the acute effects of β-AR stimulation on protein phosphorylation, sarcoplasmic reticulum (SR) Ca^{2+} handling, and inotropic/chronotropic responses. In addition we used CaMKIIδ-KO mice to examine the effects of chronic β-AR stimulation on the development of hypertrophy and maladaptive remodeling. We demonstrate that deletion of the δ-isoform of CaMKII 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. Moreover the maladaptive response to chronic β-AR stimulation, which is dissociated from its physiological and growth regulating effects, is shown to be dependent on CaMKIIδ-mediated phosphorylation of RyR2-S2814.

2.3 Results

2.3.1 Characterization of CaMKIIδ-KO mice

Adult male germline CaMKIIδ-KO mice used in this study had no obvious basal phenotype when compared to WT mice, as reported previously[22]. Data obtained from Affymetrix arrays revealed that loss of CaMKIIδ expression was not associated with compensatory mRNA upregulation of other CaMK isoforms (Fig. 2.1A). Deletion of two major splice variants of CaMKIIδ, δ_B (which contains a nuclear localization sequence) and δ_C, was confirmed by Western blot (Fig. 2.1B). 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 (Fig. 2.1C). 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.

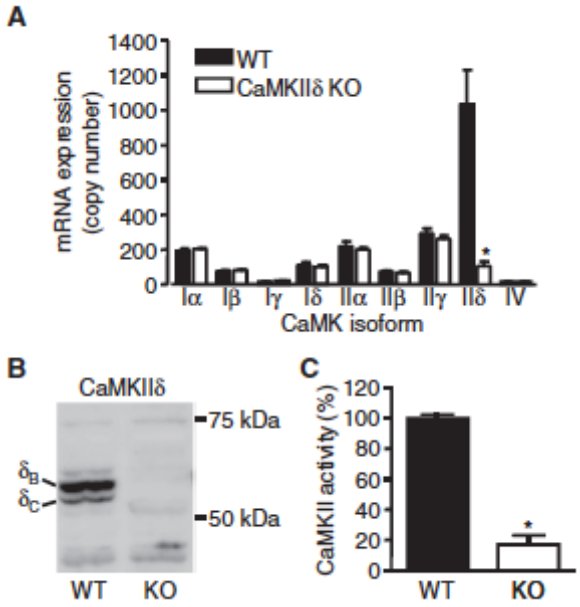

Figure 2.1. CaMK isoform expression and activity in CaMKIIδ-KO mice. **A,** mRNA transcript expression showing CaMK isoforms expressed in ventricular tissue from WT and CaMKIIδ-KO mice (n = 3 hearts each). The copy number was determined using the Affymetrix mouse gene array. **B,** Representative Western blot showing presence and absence of CaMKIIδ protein expression in left ventricular tissue isolated from WT and CaMKIIδ-KO mice, respectively. **C,** Total activity of CaMKII determined in lysates from WT and CaMKIIδ-KO hearts (n=6 each). The data are presented as the mean±SEM. *p<0.05 versus WT.

2.3.2 Deletion of CaMKIIδ reduces phosphorylation of Ca^{2+} handling proteins in adult cardiac myocytes

CaMKIIδ phosphorylates a number of cardiac proteins involved in Ca^{2+} handling, including phospholamban (PLN) and the cardiac ryanodine receptor (RyR2). CaMKII and PKA share PLN and RyR2 as targets, but the phosphorylation sites are distinct and can be phosphorylated independently by the two protein kinases[94, 95]. To

examine the effects of CaMKIIδ gene deletion on the phosphorylation of PLN and 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. The myocytes were then harvested for Western blotting (Fig. 2.2). Representative Western blots (Fig. 2.2A and D) confirmed that the total levels of expression of PLN and RyR2 were unchanged in hearts from CaMKIIδ-KO animals compared to WT controls. Myocytes from CaMKIIδ-KO mice showed ~50-60% decreases in basal phosphorylation of PLN at the CaMKII site (Thr17) and RyR2 at the CaMKII site (Ser2814) compared to WT (CTL, Fig. 2.2B and E). There was no decrease in basal phosphorylation at the PKA sites (pPLN Ser 16; pRyR Ser2808) in CaMKIIδ-KO cells (CTL, Fig. 2.2C and F). Treatment of WT mouse myocytes with 100 nmol/L ISO lead to a robust increase in phosphorylation of PLN at the CaMKII site, which was significantly attenuated in CaMKIIδ-KO cells (ISO, Fig. 2.2A and B). RyR2 phosphorylation at the CaMKII site was also increased by ISO in WT mouse myocytes and this response was significantly diminished in the CaMKIIδ-KO cells (ISO, Fig. 2.2D and E). There was no effect of CaMKIIδ deletion on phosphorylation of PLN at Ser16 or RyR at Ser2808 (Fig. 2.2C and F), consistent with these sites being regulated directly through β-adrenergic effects on PKA.

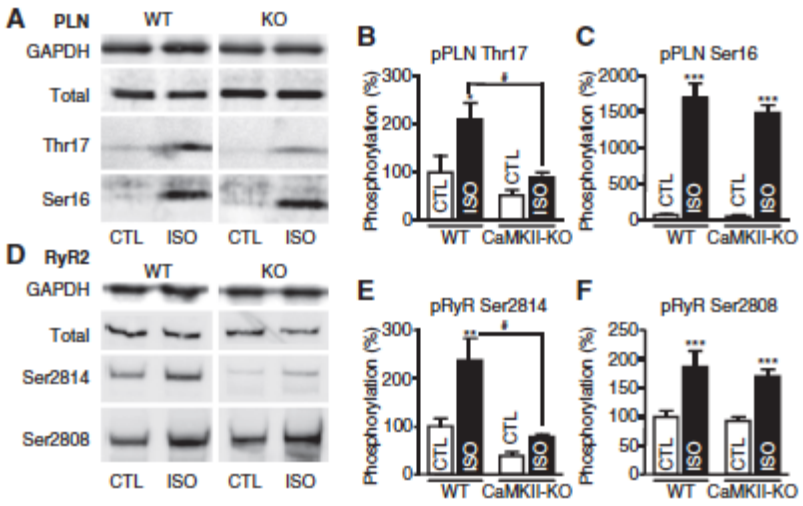

Figure 2.2. Effect of CaMKIIδ deletion on the phosphorylation of CaMKII targets in response to acute β-AR stimulation. **A,** Representative Western blot showing the phosphorylation of phospholamban (PLN) in adult mouse ventricular myocytes (AMVMs) harvested 5 min after stimulation with 100 nmol/L ISO or vehicle (CTL). **B,** Quantification of PLN phosphorylation at the CaMKII site Thr17. **C,** Quantification of PLN phosphorylation at the PKA site Ser16. **D,** Representative Western blots showing the phosphorylation of the ryanodine receptor (RyR2). **E,** Quantification of RyR2 phosphorylation at CaMKII site Ser2814. **F,** 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 vs. WT.

2.3.3 Deletion of CaMKIIδ reduces diastolic SR Ca^{2+} leak

RyR2 phosphorylation at CaMKII sites has been linked to increases in diastolic SR Ca^{2+} leak through studies carried out in CaMKII transgenic and Epac2 knockout mice, as well as in phosphomimetic and non-phosphorylatable RyR2 mutant mice[46, 96-99]. We used myocytes isolated 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 (Fig. 2.3).

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 (Fig. 2.3B). CaSpF and SR Ca^{2+} leak can be influenced by SR Ca^{2+} load (Fig. 2.3C and D), but CaSpF normalized for SR Ca^{2+} load was still increased 2.8 fold in WT and not increased significantly in CaMKIIδ-KO (Fig. 2.3E). These data are consistent with the observed changes in RyR2-Ser2814 phosphorylation (Fig. 2.2) and its dominant role in regulating SR Ca^{2+} leak, and implicate CaMKII in ISO induced dysregulation of SR Ca^{2+} homeostasis.

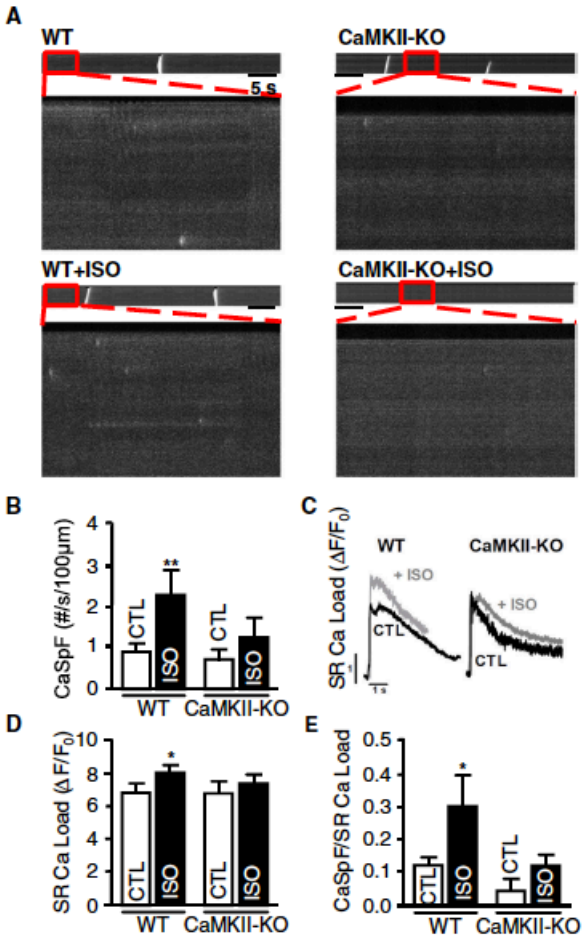

Figure 2.3. CaMKIIδ is critical for ISO-induced SR Ca^{2+} leak. **A,** Representative Ca^{2+} spark recordings from WT cardiomyocytes ± isoproterenol (ISO; 100 nmol/L) and CaMKIIδ-KO ± ISO (100 nmol/L) (represented as F/F_0 after background subtraction). Bright angled lines are Ca^{2+} waves and regions in red boxes are expanded to show sparks. **B,** 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). **C,** Representative caffeine evoked Ca^{2+} transients (SR Ca^{2+} load) for WT (left) and CaMKIIδ-KO (right) in control condition (black) and in the presence of ISO (grey). **D,** Mean SR Ca^{2+} loads determined as in C for WT±ISO (CTL: n=5 vs. ISO: n=7) and CaMKIIδ-KO±ISO (CTL: n=7 vs. ISO: n=10) from same animals as in B. **E,** CaSpF normalized to SR Ca^{2+} load in same cells as C-D. *p<0.01 vs. CTL; **p<0.001 vs. CTL (Student's t test).

2.3.4 Fight-or-flight response to acute β-AR stimulation is not altered in CaMKIIδ KO mice

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 as described[100]. We first used an enzymatic CaMKII activity assay to demonstrate that ISO treatment of isolated hearts activates CaMKII, as assessed by increases in the percent of autonomous (Ca^{2+} independent) activity. Indeed autonomous CaMKII activity was increased more than 2-fold (from 9 to 22% of total activity) at 10 min after addition of 1 μmol/L ISO (Fig. 2.4A). Isolated heart function was studied at a fixed left ventricular end diastolic pressure of 10 mmHg. Baseline isovolumic cardiac performance was identical in isolated hearts from WT and CaMKIIδ-KO mice, and ISO increased the rate and magnitude of pressure generation and the rate of pressure decay in hearts from both WT and CaMKIIδ-KO animals. Representative baseline left ventricular dP/dt recordings and ISO responses are shown (Fig. 2.4B). Left ventricular developed pressure (LVDP) was increased by 43.8% in WT and to a comparable extent (44.8%) in CaMKIIδ-KO (Fig. 2.4C). This agrees with the preserved β-AR-induced inotropic and lusitropic effects seen in CaMKIIδ-KO myocytes[101]. The increase in heart rate (40.0% in WT, 45.8% in CaMKIIδ-KO) was also not different (Fig. 2.4D).

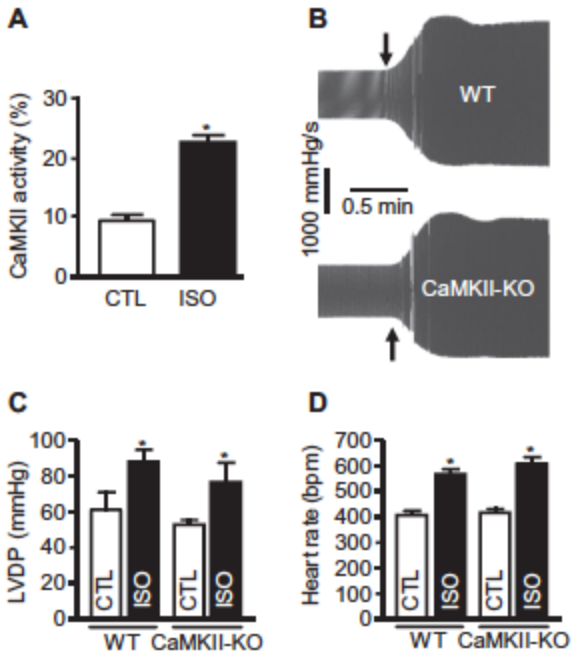

Figure 2.4. Effects of acute ISO exposure (1 μmol/L) on CaMKII activity, contractility and spontaneous beating rate of isovolumic Langendorff perfused hearts. **A,** Autonomous CaMKII activity measured at steady state under control (CTL) conditions, and after 10 minutes of ISO perfusion. Activity of CaMKII was determined in fresh ventricular lysates from WT mouse hearts. **B,** Representative, simultaneously recorded dP/dt (mmHg/s) tracings showing changes of the rate of pressure development from steady-state baseline to maximum during ISO infusion. The arrows indicate start of ISO infusion. **C,** Group averages of left ventricular developed pressure (LVDP). **D,** Group averages of spontaneous beating rate. Data are presented as mean±SEM. n = 6 mice per group; *p<0.05 vs. CTL.

2.3.5 Deletion of CaMKIIδ has no effect on cardiac hypertrophy

The hypertrophic response of the mouse heart to β-AR stimulation with ISO is well described[102-104]. 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.

Wild-type and CaMKIIδ-KO littermates were treated ISO for 9 days (Fig. 2.5A; 15 mg/kg/day i.p.), 2 weeks (Fig. 5B; 30 mg/kg/day s.c. infusion), or 4 weeks (Fig. 5C; 10 mg/kg/day i.p.). Wild-type mice exhibited significant increases in left ventricular mass in response to ISO. Daily injections of ISO for 9 days or 4 weeks led to 27 and 35% increases in heart weight to body weight ratio respectively, as compared to isovolumic vehicle (sterile saline) (Fig. 2.5A and C). Infusion of ISO resulted in a 28% increase in heart weight to body weight ratio as compared to control (Fig. 2.5B). Notably, CaMKIIδ-KO hearts showed increases in heart/body weight that were equivalent to those of WT with all times and routes of ISO administration. Three other measures of cardiac hypertrophy were also assessed. Phosphorylation of the class II histone deacetylase, HDAC-5, at serine 498 (which enables transactivation of hypertrophic genes) was significantly increased by ISO treatment in both CaMKIIδ-KO mice and WT littermates (Fig. 2.5D). Atrial natriuretic peptide (ANP) showed significantly increased expression following ISO treatment in WT mice and this was not attenuated in the CaMKIIδ-KO (Fig. 2.5E).

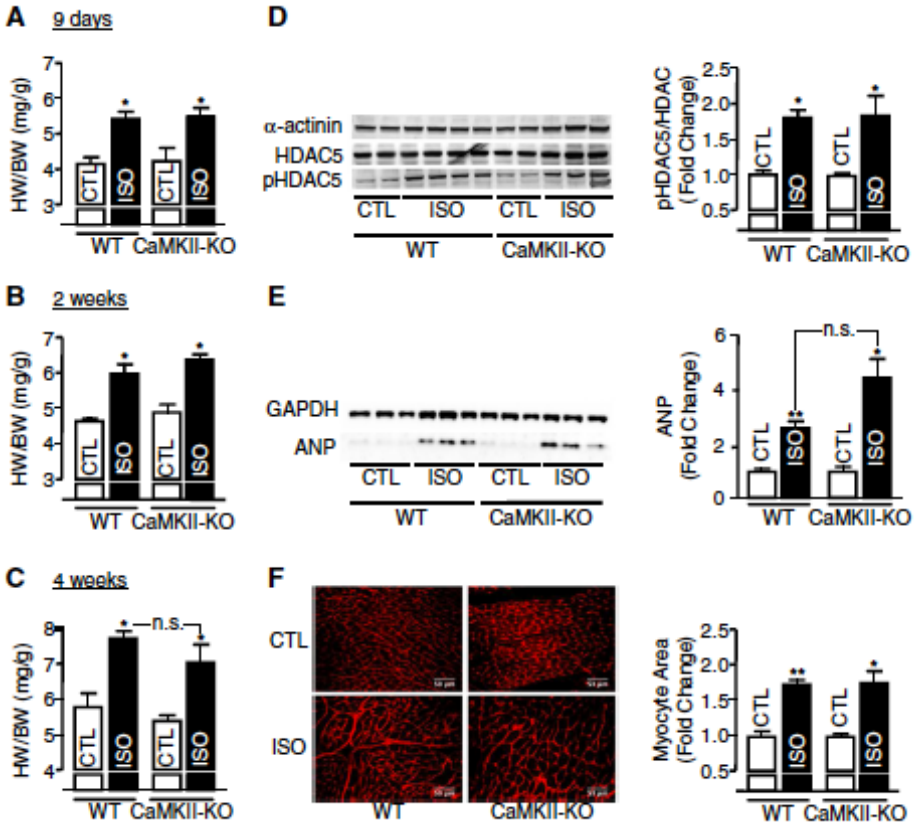

Figure 2.5. CaMKIIδ deletion does not inhibit cardiac hypertrophy in response to chronic β-AR stimulation (n = 5-9 per group). **A-C,** Heart weight normalized to body weight (HW/BW) of WT and CaMKIIδ-KO mice after 9 days (15 mg/kg/d i.p.), 2 weeks (30 mg/kg/d s.c. infusion), and 4 weeks (10 mg/kg/d i.p.) of continuous ISO treatment. **D,** Representative Western blot of HDAC5 phosphorylation at Ser498 in WT and CaMKIIδ-KO hearts after 9 days of once-daily 15 mg/kg ISO injections. Quantification is shown to the right and is presented as the ratio of normalized phosphorylated HDAC5 over total HDAC5 (n=3-4 per group). **E,** Representative Western blot showing ANP protein expression in both WT and KO mice after 2 weeks of 30 mg/kg/d ISO infusion. Quantification is shown to the right (n=3 per group). **F,** Representative photographs of hearts from WT and KO mice stained with TRITC-labeled wheat germ agglutinin following 4 weeks of once-daily 10 mg/kg ISO injection (n=3 per group). Quantification is shown to the right (300 cells counted per group). Data are presented as mean±SEM; *p<0.05 vs. CTL; **p<0.01 vs. CTL.

The phosphorylation of Akt was determined in the same tissue samples and shown to not be increased by chronic ISO infusion in either WT or KO hearts (Fig. 2.6) thus there is no evidence for development of a "physiological" hypertrophy in response to chronic ISO treatment.

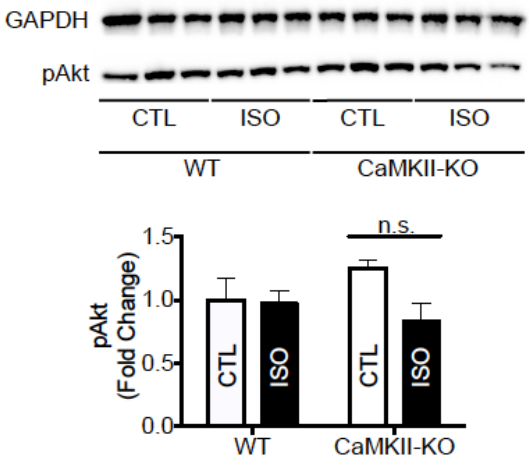

Fig. 2.6. Chronic infusion of ISO does not increase the phosphorylation of Akt. Western blots of whole heart lysates from mice infused with 30 mg/kg/d isoproterenol for 2 weeks. Quantification of Akt phosphorylation at Ser473 is shown to the right. Data are presented as mean±SEM of the ratio of normalized phosphorylated Akt over total Akt (n=3 per group).

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 other evidence for unchanged pathological hypertrophy at the cellular level (Fig. 2.5F). The finding that the hypertrophic response to β-adrenergic stimulation is independent of CaMKIIδ activation extends and is consistent with our previous observations that both pressure

overload (TAC) and cardiac Gαq expression induce cardiac hypertrophy independent of CaMKIIδ[22, 36].

2.3.6 CaMKII deletion inhibits fibrosis induced by chronic β-AR stimulation

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 (Fig. 2.7A). 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 (Fig. 2.7B).

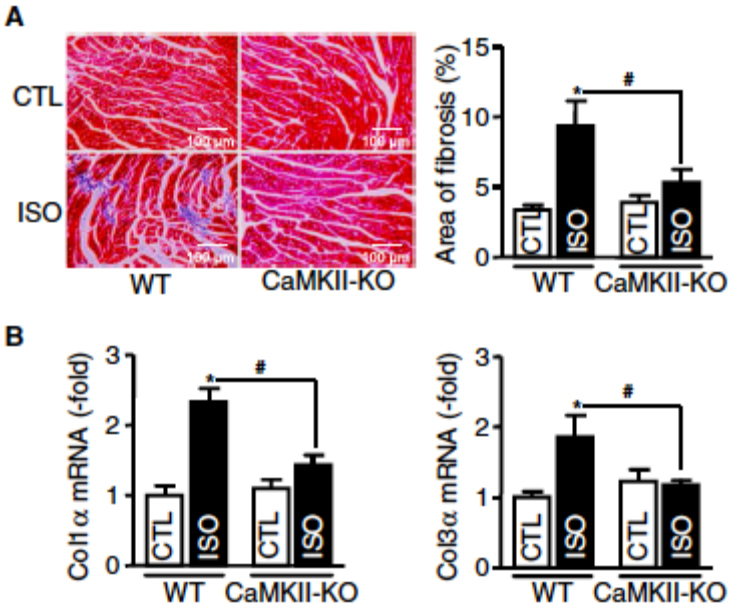

Figure 2.7. CaMKIIδ deletion inhibits cardiac fibrosis after 2-week continuous ISO infusion. **A,** Representative photographs of hearts stained with Masson's trichrome show collagen deposition, indicative of cardiac fibrosis (left). Quantification of cardiac fibrosis (right, n=4). **B,** Pro-collagen I (left) and III (right) mRNA expression in hearts from WT and CaMKIIδ-KO mice (n=3 per group) after 2-week ISO infusion. Data are presented as mean±SEM; *p<0.05 vs. CTL; #p<0.05 WT vs. KO.

To rule out the possibility that the marked difference in development of fibrosis resulted from loss of CaMKII signaling in fibroblasts, we also carried out studies using mice in which CaMKIIδ was selectively deleted in cardiac myocytes. The cardiac-specific CaMKIIδ-KO (CKO) mice were generated by crossing heterozygous floxed CaMKIIδ mice with MLC 2v Cre mice. At baseline the CKO mice showed normal ventricular chamber dimension, wall thickness and cardiac function as assessed by echocardiography[11]. Using the cardiac specific CaMKIIδ-KO mouse we

confirmed that CaMKIIδ deletion significantly impairs development of fibrosis in response to 4-week injection of ISO (Fig. 2.8).

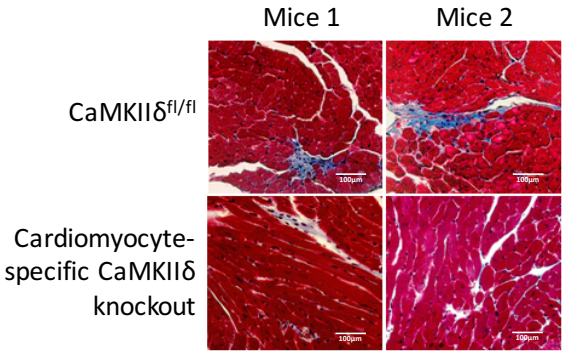

Fig. 2.8. Cardiomyocyte-specific CaMKIIδ deletion attenuates cardiac fibrosis induced by 4 weeks ISO injection. Masson's Trichrome staining showing collagen deposition (blue) in hearts from ISO treated WT mice (top) and protection from fibrosis in cardiac specific CaMKIIδ-KO hearts (bottom).

Thus, the CaMKIIδ-mediated β-AR signal that induces fibrosis appears to be cardiomyocyte autonomous. These findings suggest that CaMKIIδ activation in cardiomyocytes contributes to the development of interstitial tissue abnormalities that accompany the progression of heart failure in the setting of chronic β-adrenergic stress.

2.3.7 CaMKII deletion protects from heart failure development induced by sustained β-AR stimulation

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 (Fig. 2.9A). 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 (Fig. 2.9B). The CaMKIIδ-KO mice subjected to ISO also showed a trend towards improved survival compared with WT (Fig. 2.9C). Fibrosis was significantly increased in hearts from WT mice injected with ISO for 4 weeks and this response was attenuated by CaMKIIδ deletion (Fig. 2.9D).

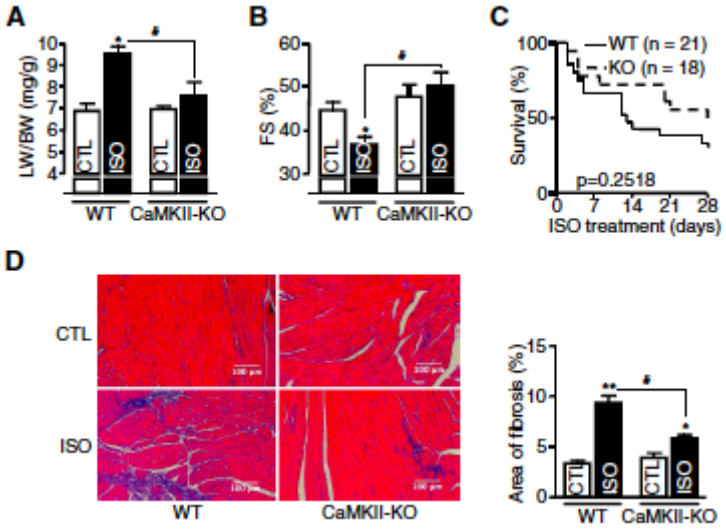

Figure 2.9. Effect of CaMKIIδ deletion on the development of heart failure and cardiac fibrosis after 4-weeks of daily ISO injection. **A,** Lung weight normalized to body weight (LW/BW) of WT and CaMKIIδ-KO mice (n=7-9 per group). **B,** Left ventricular fractional shortening (FS). **C,** Kaplan-Meier survival curves of WT and CaMKIIδ-KO mice treated with ISO or vehicle. **D,** Representative photographs of hearts stained with Masson's trichrome show collagen deposition, indicative of cardiac fibrosis (left). Quantification of cardiac fibrosis is shown to the right (n=3 per group). *p<0.05 vs. CTL; **P<0.01 vs. CTL; #p<0.05 WT vs. KO.

2.3.7 Inactivation of the CaMKII phosphorylation site RyR2 inhibits development of heart failure induced by chronic β-AR stimulation

To determine whether CaMKII-mediated phosphorylation of RyR2 contributes to the development of ISO-induced heart failure we examined the responses to chronic ISO in S2814A knock-in mice in which the major CaMKII phosphorylation site on RyR2 was genetically inactivated. These mice were previously shown to exhibit reduced cardiac remodeling and delayed progression of heart failure after TAC[98]. Studies were carried out using a protocol in which mice were subjected to continuous infusion of ISO (20 mg/kg/day) for a 4-week period. Echocardiographic measurements were carried out at various times following the ISO infusion protocol. Comparing 1 and 4 weeks of ISO treatment revealed that ISO treatment led to significant decreases in contractile function (Fig. 2.10A-D) and to development of cardiac dilation (Fig. 2.10E and F) in WT mice that were not significant in the S2814A mice.

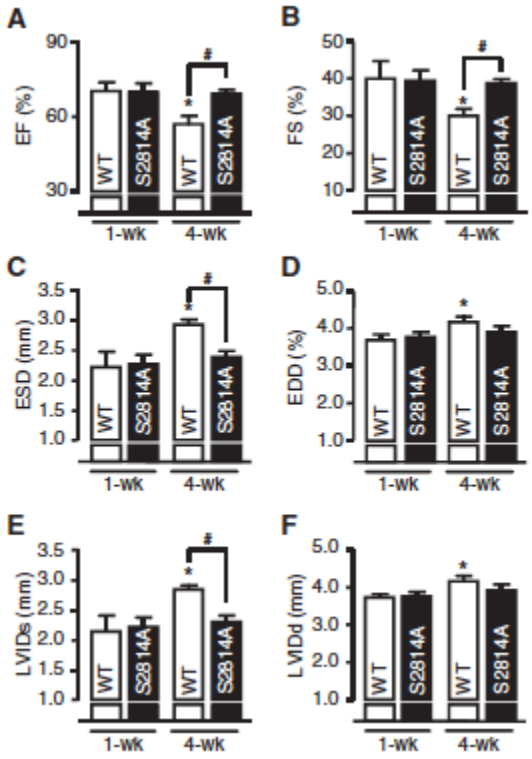

Figure 2.10. Echocardiographic parameters of wild-type and S2814A mice at one (1-wk) and four (4-wk) weeks of chronic ISO infusion. Data are presented as mean±SEM. **A,** EF = ejection fraction; **B,** FS = left ventricular fractional shortening; **C,** ESD = end-systolic diameter; **D,** EDD = end-diastolic diameter; **E,** LVIDs = systolic left ventricular interior diameter; **F,** LVIDd = diastolic left ventricular interior diameter. *p<0.05 vs. 1-week WT (one-tailed t test); #p<0.01 WT vs. S2814A (two-tailed t test).

2.4 Discussion

Our prior studies demonstrated that transgenic overexpression of the predominant cardiac isoform of CaMKII, CaMKIIδ, is sufficient to induce heart failure[46]. We subsequently reported that genetic deletion of CaMKIIδ in mice did not inhibit pressure overload-induced hypertrophy but limited the transition from hypertrophy to heart failure[22]. Further

studies established that CaMKIIδ deletion also protected the heart against *in vivo* ischemia-reperfusion damage and conferred improved functional recovery[11]. In the present study we examined the role of CaMKIIδ in responses to both acute and chronic β-AR stimulation. Acute β-AR activation effects on contractility and heart rate were unaltered in CaMKIIδ-KO mice. This is consistent with previous data using isolated myocytes and demonstrating comparable inotropic and lusitropic responses to ISO in CaMKIIδ-KO compared to WT cells[101]. Chronic ISO-induced stress also resulted in comparable cardiac hypertrophy in WT and CaMKIIδ-KO mice. Recent studies from the Backs laboratory using a double knockout in which CaMKIIγ, a more minor cardiac isoform, was genetically deleted along with CaMKIIδ confirmed that hypertrophy in response to TAC or ISO were not CaMKII dependent[105]. Indeed they observed greater hypertrophy in the CaMKII double knockout, due to diminished inhibition of the calcineurin pathway.

Strikingly however, the development of cardiac fibrosis and progression to heart failure were inhibited in CaMKIIδ-KO subject to chronic treatment with ISO, consistent with what we observed when these mice were subject to TAC[22]. More specifically, involvement of CaMKII in the progression of hypertrophy to failure following 6 week TAC[22] or 4 week ISO injection was evidenced by improved fractional

shortening, less increase in lung/body weight, and attenuated cardiac fibrosis. 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 (only one third of the WT mice survived the 4 week ISO injection) and the somewhat diminished mortality seen in the CaMKIIδ-KO mice (half survived the 4 week ISO injection). 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. It has been reported that daily injections of ISO result in higher peak levels of ISO and greater hemodynamic stress than does ISO infusion[103]. The cardiomyopathic effects of ISO treatment also vary with the strain of mice being used[102, 106]. Differences in the genetic background of the RyR2814 versus CaMKII KO, as well as the use of 4 week ISO infusion (vs. injection) in studies using the RyR2814 mutant and WT mice, may explain why no mortality was seen in these mice.

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[91, 98, 107]. CaMKII phosphorylates RyR2 at serine 2814 (S2814) and increases the open probability of the channel, irrespective of changes in SR filling, which may also be affected in failing hearts[108]. In our studies on TAC induced hypertrophy we demonstrated that phosphorylation of RyR2 at the CaMKII site and the associated Ca^{2+} leak were significantly increased at 6 week TAC and suggested that this could be a molecular mechanism contributing to the transition to heart failure. Further evidence that CaMKII-mediated RyR2 phosphorylation is critical for TAC induced heart failure development was provided by studies demonstrating that RyR2 S2814A mutant mice had significantly better cardiac contractility and exhibited less fibrosis than WT mice at 4 months after TAC[98].

Studies with intact rabbit ventricular myocytes demonstrated at the cellular level that β-adrenergic receptor agonists increase SR Ca^{2+} leak independent of PKA, whereas inhibitors of CaMKII (KN-93, or autocamtide-2-related inhibitory peptide II) largely prevent the ISO-induced enhancement of SR Ca^{2+} leak[109]. This is consistent with the finding that CaMKII mediated phosphorylation of Ser2814 of RyR2 plays a predominant role in Ca^{2+} release from isolated SR vesicles after β-AR stimulation[110]. The current work extends these findings to the in vivo level

demonstrating that the ability of chronic β-AR stimulation to elicit cardiac dilatation and reduced contractility is significantly diminished in S2814A knock-in mice. These data implicate CaMKII-mediated phosphorylation of RyR2 in the response to enhanced sympathetic tone and chronic β-AR stimulation, well known stress-induced regulators of cardiac dysfunction.

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[111, 112]. Elevated mitochondrial Ca^{2+} increases production of reactive oxygen species (ROS) which can also lead to posttranslational modifications in CaMKII that render it constitutively active[19]. 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[113]. CaMKII also has numerous other targets that could contribute to heart failure development including activation

of cardiomyocyte NF-κB signaling and inflammation and phosphorylation of ion channels and myofilament proteins[11, 86].

Our previous findings showed that CaMKIIδ deletion inhibited apoptosis and associated cardiac fibrosis after 6 weeks of TAC[22] and CaMKII has been previously shown to mediate cardiomyocyte death in response to β-adrenergic stimulation[31, 33, 43, 114, 115]. We were, however, unable to document CaMKII-dependent changes in TUNEL staining in hearts from 2 week infused or 4 week injected mice. Thus while chronic ISO treatment could lead to CaMKII-mediated apoptosis and replacement fibrosis, a growing body of evidence suggests that viable cardiomyocytes, under stressed conditions, generate signals that promote interstitial fibrosis. For example, ATP release through pannexin 1 channels in cardiomyocytes contributes to the transformation of fibroblasts to profibrotic myofibroblasts following myocardial infarction[116]. Similarly, the fibrosis that occurs in pressure overload appears to require TGFβ-receptor signaling in cardiomyocytes, as it is attenuated by cardiomyocyte-specific knockout of this receptor[117]. The histological analysis shown in the current study using the cardiac specific CaMKIIδ-KO suggests that CaMKII localized in cardiomyocytes induces changes necessary for development of interstitial fibrosis during chronic β-AR stimulation (Suppl. Fig. 2). β-AR stimulation has been shown to

increase TGFβ_1 mRNA transcription and latent TGFβ_1 release in cardiomyocytes[118, 119] thus CaMKII could stimulate fibrosis through effects on generation of this or other potent profibrotic factors.

β-AR blockade is an established therapeutic which inhibits excessive β-AR signaling and prevent worsening in left ventricular function following myocardial infarction[72]. Transgenic mice overexpressing a cardiac-specific peptide inhibitor of CaMKII, AC3-I, were shown to be significantly resistant to myocardial infarction-induced left ventricular dysfunction and dilatation[90]. The same study confirmed the beneficial effects using KN-93, a pharmacological inhibitor of CaMKII, and observed that the beneficial effect of CaMKII inhibition mimic salutary effects of β-AR blockers after myocardial infarction, and were independent of heart rate, because KN-93 did not reduce heart rate in their experiments[90]. The studies presented here use genetic deletion of CaMKIIδ to provide direct evidence that CaMKII mediates the maladaptive effects of increased β-AR stimulation. Moreover results obtained using RyR2 S2814A mutant mice suggest a mechanism through which this occurs. 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.

2.5 Experimental Procedures

Black Swiss mice in which the predominant cardiac isoform, CaMKIIδ, was genetically deleted (conventional CaMKIIδ-KO mice) were generated in our laboratory and characterized as described[22]. Cardiac-specific CaMKIIδ-KO mice were generated by crossing heterozygous floxed CaMKIIδ mice with MLC2v-Cre mice to ablate the CaMKIIδ gene in ventricles as described[11]. Generation of C57/Bl6 RyR2-S2814A knock-in mice (S2814A) in which the CaMKII phosphorylation site was genetically inactivated has been described[120]. None of these mouse models showed gross baseline changes in ventricular structure or function compared to their respective wild type (WT) littermates. All mice used in the present study were male at 8-12 weeks of age, unless otherwise noted. Animal studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of University of California at San Diego and Davis, and Baylor College of Medicine.

2.5.1 Gene array and quantitative PCR

RNA extraction for microarray and Real time analysis were 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). A preoptimized Taqman Gene Expression Assays was used to quantify Col1a, Col3a, and glyceraldehyde-3-phosphate dehydrogenase mRNA as described by the manufacturer (Applied Biosystems). Gene expression was determined using Taqman® Universal PCR master mix, FAM™-labeled Taqman® probe for collagen 1a and 3a and VIC™-labeled Taqman® probe for GAPDH (Applied Biosystems). Microarray analysis was done using the GeneChip Mouse Gene 1.0 ST Array and an GeneChip system comprising a Hybridization Oven 320, a GeneChip Fluidics Station 450, and a high-resolution GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

2.5.2 Isolation of adult mouse ventricular myocytes

Cardiac ventricular myocytes were isolated from the ventricles of 8–16-weeks-old WT or CaMKIIδ-KO mice. Animals were anesthetized with pentobarbital, 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 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 hr, cells were washed and serum-free medium was added back.

2.5.3 Line Scan Confocal Microscopy

Cells were isolated with the retrograde Langendorff perfusion technique as previously described[97]. 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 (BioRad, Radiance 2100, x40 oil immersion objective) in line scan mode (3 ms/line). Excitation was via Ar laser (488 nm) and emission was collected at >505 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.

2.5.4 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 phosphocellulose paper. Percent activation was calculated as the ratio of autonomous to maximal activity for each sample.

2.5.5 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 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. The left atrium was removed, and a water-filled balloon connected to a pressure transducer (MLT844, ADInstruments) was inserted into the left ventricle and inflated to 10 mmHg of LV end-diastolic pressure (LVEDP). Pre-agonist baseline data were recorded following a 20-min equilibration period. Subsequently, an ISO (1 μmol/L) infusion was initiated, and peak response 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 nitrogen and stored at -80 °C until processed. For assay of CaMKII activity hearts were homogenized and assayed immediately.

2.5.6 Histological analysis

Cardiac left ventricles were fixed for 24 hr 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. The area of fibrosis was quantified according to the method by Gaspard and Pasumarthi[121]. Briefly, 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 fibrosis 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).

2.5.7 Immunoblotting

Western blot analysis from snap frozen ventricular tissue was performed according to protocols described previously[11]. The antibodies for immunoblotting were as follows: CaMKIIδ (D.M. Bers, UC Davis); PLN and a-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); Ser498 pHDAC5 (Assay Biotech).

2.5.8 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 isoproterenol 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 interaction 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.

2.5.9 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 carried out in the Wehrens laboratory at Baylor College of Medicine using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described[122]. 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. Heart rate varied per mouse in response to isoproterenol.

2.5.10 Statistics

Results are presented as mean ± SEM, and comparison between groups was performed using ANOVA unless otherwise indicated. All statistical analyses were performed using Microsoft Excel software. A p-value < 0.05 was considered statistically significant.

2.6 Acknowledgements

Chapter 2 with minor modifications was reprinted from the *Journal of Molecular and Cellular* Cardiology, volume 85 by Grimm M., Ling H., Willeford A., Pereira L., Gray C.B.B., Erickson J.R., Sarma S., Respress J.L., Wehrens X.H.T., Bers D.M., Brown J.H., "CaMKIIδ mediates β–adrenergic effects on RyR2 phosphorylation and SR Ca^{2+} leak and the pathophysiological response to chronic β–adrenergic stimulation." Pages 282-291, copyright 2015. This work was supported by NIH grants P01 HL080101-05 (to JHB and DMB). HL was supported by an American Heart Association Post-Doctoral Fellowship (0825268F). CG was, and AW is currently, supported by 2T32-GM007752. JLR was supported by T32-HL07676 and LP was supported by an AHA postdoctoral fellowship. Other sources of support include NIH grant HL28143 to JHB, the Foundation Leducq (Alliance for CaMKII Signaling in Heart) to XHTW and DMB, and

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Chapter 3

Cardiomyocyte CaMKIIδ initiates angiotensin II-induced inflammatory responses and fibrosis

3.1 Summary

CaMKIIδ mediates development of cardiac fibrosis and decompensation to heart failure in response to multiple stressors. The underlying mechanism is not known. Fibrosis and heart failure are strongly associated with cardiac inflammation. We postulated that it is the activation of CaMKIIδ in cardiomyocytes which triggers inflammatory gene expression and recruitment of inflammatory cells leading to adverse remodeling in response to hypertensive stimuli. The objective of this work was to determine whether activation of CaMKIIδ in cardiomyocytes serves to initiate inflammatory gene expression in

response to infusion of angiotensin II (Ang II), a hypertensive non-ischemic stress, and demonstrate that this contributes to macrophage recruitment and development of fibrosis. Studies were carried out in control (CaMKIIδ$^{fl/fl}$) mice and mice lacking CaMKIIδ in cardiomyocytes (CKO). Ang II infusion for 1 day led to robust induction of mRNA for multiple pro-inflammatory chemokines and cytokines which was markedly attenuated in CKO mouse hearts. Macrophage recruitment was also significantly diminished. The effect of CaMKIIδ deletion was not associated with alterations in cell death or blood pressure responses. Cardiomyocyte expression of monocyte chemotactic protein 1 (MCP-1) mRNA and ventricular expression of MCP-1 protein induced by Ang II infusion were diminished in the CKO mouse heart, as was priming and activation of the inflammasome. CaMKIIδ expression in cardiomyocytes *in vitro* also elicited inflammasome priming and activation which involved reactive oxygen species. Inhibiting MCP-1 or inflammasome signaling attenuated Ang II-induced inflammatory gene expression and macrophage recruitment. In addition, the robust cardiac fibrosis elicited by Ang II at longer times was significantly diminished by these inhibitors and by deletion of CaMKIIδ in cardiomyocytes. In conclusion, we show that a cardiomyocyte autonomous transcriptional pathway triggered by CaMKIIδ signaling is necessary for Ang II-mediated inflammatory gene

expression and promotes MCP-1 and inflammasome dependent macrophage recruitment and fibrosis.

3.2 Introduction

Cardiac hypertrophy is induced by diverse disease-related stresses including hypertension and is considered to be an initial adaptive response, normalizing ventricular wall stress and maintaining cardiac output. When stress is sustained, however, adverse cardiac remodeling and eventual heart failure ensue[123-125]. Despite intensive research efforts our understanding of the mechanisms responsible for decompensation to heart failure remains incomplete. There is considerable evidence indicating that inflammation is related to the development of fibrosis, a key factor in adverse remodeling[38-41]. In addition, systemic inflammation is strongly associated with human heart failure[48-50]. While attempts to use inflammatory mediators as therapeutic targets has met with limited success[48, 49], further understanding of the basic early mechanisms triggering inflammation could provide new approaches to the development of efficacious anti-inflammatory interventions.

The initiating causes of cardiac inflammation in the context of ischemic insults (myocardial infarction/ischemia reperfusion) have been the focus of considerable research. Cell death (especially necrotic cell death) leads to release of intracellular molecules such as ATP and

HMGB1, referred to as damage-associated molecular patterns (DAMPS)[52, 53]. These act on various cardiac cells to stimulate pro-inflammatory transcriptional pathways, increasing expression and release of cytokines and chemokines[54-60]. Inflammation also occurs, however, in models of non-ischemic heart disease where cell death is not robustly induced. Angiotensin II (Ang II) infusion is the most common model used to study inflammatory and fibrotic changes induced by a hypertensive non-ischemic stress. This treatment leads to robust induction of chemokines and cytokines and subsequent recruitment of monocytes/macrophages to the heart[38, 61, 64-66, 70]. Recent studies have also demonstrated that pressure overload leads to inflammatory responses and recruitment of immune cells that can contribute to adverse cardiac remodeling[62, 67-69, 71]. Notably, the molecular mechanism by which inflammation is initiated in the heart in response to hypertensive stresses such as infusion of angiotensin II or pressure overload is not known.

Our previous studies using mice in which the calcium/calmodulin-dependent protein kinase II δ (CaMKIIδ) was genetically deleted demonstrated that CaMKIIδ signaling is not required for the development of cardiac hypertrophy[22, 35, 36]. On the other hand, CaMKIIδ clearly plays a prominent role in adverse cardiac remodeling, affecting development

of fibrosis and the transition to heart failure in response to pressure overload, β-adrenergic receptor activation, Gαq overexpression, and myocardial infarction[20-22, 35, 36]. Our lab and others have also demonstrated that CaMKIIδ contributes to inflammatory gene expression through activation of nuclear factor kappa B (NFkB), at least in the context of ischemic injury[10-12]. In this study, we test the hypothesis that CaMKIIδ, activated in cardiomyocytes through non-ischemic stress, contributes to adverse remodeling by initiating inflammatory gene expression, macrophage recruitment, and fibrosis.

A major player in inflammation is the NOD-like pyrin domain-containing protein 3 (NLRP3) inflammasome, a multiprotein complex that regulates maturation of the proinflammatory cytokines interleukin 1β (IL-1β) and interleukin 18 (IL-18) through activation of caspase 1[126]. Although the role of inflammasomes in cardiac pathophysiology has not been extensively examined there is recent evidence for inflammasome activation in the heart under ischemic conditions induced by myocardial infarction and ischemia/reperfusion[127-131]. Thus, we further determined whether non-ischemic hypertensive stress regulated by Ang II activates the NLRP3 inflammasome and whether this signaling event is regulated by CaMKIIδ signaling in cardiomyocytes.

In the studies reported here we use cardiomyocyte-specific CaMKIIδ knockout (CKO) mice to demonstrate that 1 day Ang II infusion elicits robust increases in proinflammatory cytokine and chemokine mRNA which depend on activation of CaMKIIδ in cardiomyocytes. We demonstrate that the chemokine monocyte chemotactic protein 1 (MCP-1/CCL2) is induced in cardiomyocytes through CaMKIIδ and contributes to recruitment of macrophages and to subsequent development of fibrosis. In addition, we show CaMKIIδ-dependent activation of the NLRP3 inflammasome in the heart and provide evidence for its role in Ang II-induced inflammatory and pro-fibrotic signaling. Our findings provide evidence that acute CaMKIIδ activation in cardiomyocytes can initiate cardiac autonomous inflammatory responses that significantly contribute to inflammatory cell recruitment and development of fibrosis in the heart.

3.3 Results

3.3.1 Angiotensin II infusion activates CaMKIIδ and NFkB

To demonstrate that infusion of Ang II activates cardiac CaMKIIδ, osmotic mini-pumps were used to deliver Ang II at 1.5 μg/kg/min, the standard protocol used by others[38, 66, 132]. Hearts were isolated 1 and 3 hours after the start of infusion and activation of CaMKII assessed using a ^{32}P enzymatic CaMKII activity assay. Activated CaMKIIδ was increased

by 1.3 and 1.6-fold at 1 and 3 hours of infusion, respectively (Fig. 3.1A). NFkB activation was assessed by immunoblotting for increases in the p65 NFkB subunit in nuclear fractions. Nuclear p65 was increased in control (CaMKIIδ$^{fl/fl}$) mouse hearts after 3 hr Ang II infusion and this response was significantly attenuated in hearts from cardiomyocyte-specific CaMKIIδ KO mice, (Fig. 3.1B). Taken together these data support previous evidence that Ang II activates CaMKIIδ[19, 84, 85] and extend previous reports that activated CaMKIIδ contributes to NFkB regulation[11, 12].

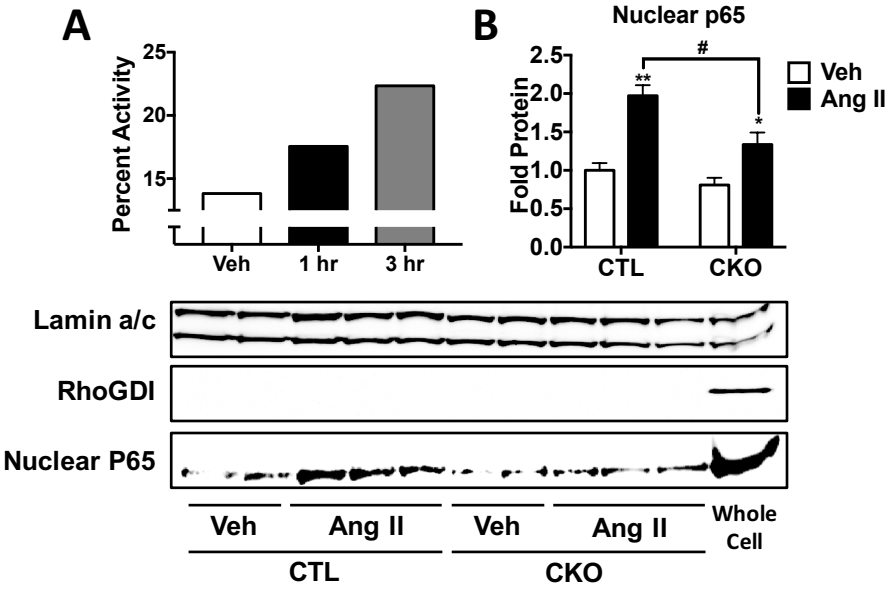

Figure 3.1. Angiotensin II infusion activates CaMKIIδ and elicits NFkB signaling through CaMKIIδ. **A,** Autonomous CaMKII activity measured in ventricular lysates at baseline and after 1 and 3 hours of Ang II infusion. **B,** Western blot and quantitation of the nuclear factor kappa B subunit P65 in nuclear fractions isolated from hearts of control (CTL) and cardiomyocyte-specific CaMKIIδ knockout (CKO) mice after 3 hours of Ang II infusion (n=2-3 each group). Lamin a/c was used as a nuclear loading control and RhoGDI was used as a nuclear fraction purity control. *P<0.05 vs Veh, **P<0.01 vs Veh, #P<0.05 CTL vs CKO.

3.3.2 CaMKIIδ activation in cardiomyocytes mediates angiotensin II-induced cardiac inflammatory chemokine and cytokine expression

To determine the involvement of cardiomyocyte CaMKIIδ in Ang II-induced inflammatory gene expression, control and cardiomyocyte-specific CaMKIIδ KO mice were infused with 1.5 μg/kg/min Ang II. Hearts were isolated after 1 day Ang II infusion and lysates used for preparation of mRNA. Robust increases in expression of mRNA for monocyte chemotactic protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1α (MIP1α/CCL3), C-X-C motif ligand 1 (CXCL1), C-X-C motif ligand 2 (CXCL2), interleukin 1β (IL-1β), and interleukin 6 (IL-6) were observed in control hearts. Remarkably, these responses were attenuated by 70-90 percent in hearts from CKO mice (Fig. 3.2A). Increases in mRNA levels of MCP-1, IL-1β and IL-6 were detectable by as early as 3 hours Ang II infusion and were also markedly dependent on the presence of CaMKIIδ (Fig. 3.3).

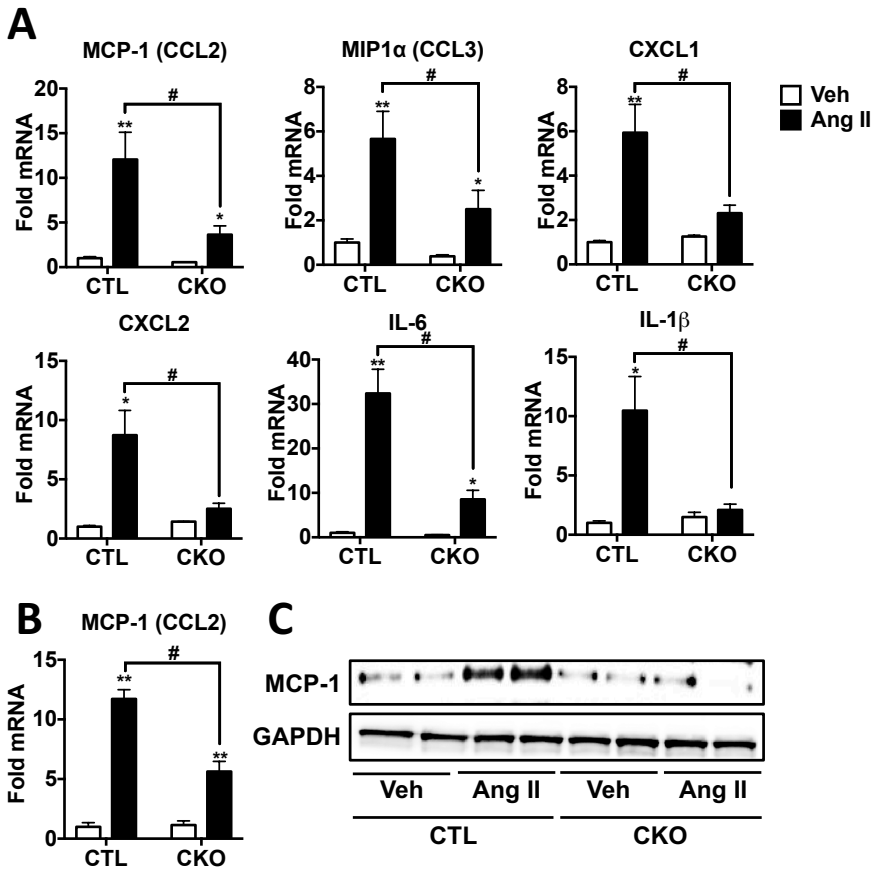

Figure 3.2. Deletion of CaMKIIδ in the cardiomyocyte attenuates Ang II-induced inflammatory gene expression in the heart and monocyte chemotactic protein 1 expression in the myocyte. **A,** mRNA expression of inflammatory chemokines and cytokines in ventricles of control and CKO mice infused with Ang II (1.5 μg/kg/min) for 1 day as measured by qPCR (n=4-6 each group). **B,** MCP-1 mRNA expression in cardiomyocytes isolated from control and CKO mice after 1 day Ang II as measured by qPCR (n=3 each group). **C,** Western blot of MCP-1 protein in ventricular lysates of control and CKO mice after 1 day Ang II infusion (n=4 each group). **P*<0.05 vs Veh, ***P*<0.01 vs Veh, #*P*<0.05 CTL vs CKO.

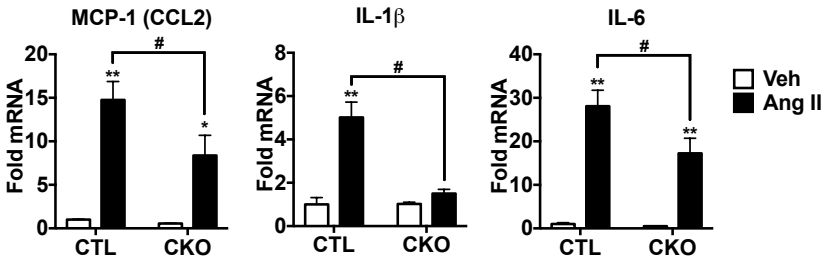

Figure 3.3. Angiotensin II increases cardiac inflammatory gene expression through cardiomyocyte CaMKIIδ at 3 hours infusion. mRNA expression of inflammatory chemokines and cytokines in the ventricles as measured by qPCR (n=3-6 each group). *P<0.05 vs Veh, **P<0.01 vs Veh, #P<0.05 CTL vs CKO.

MCP-1 has been shown to play a role in Ang II-induced inflammation and fibrosis, with the source of MCP-1 upregulation and generation thought to be endothelial cells[66, 80, 133]. To demonstrate that the cardiomyocyte can also act as a source of MCP-1 expression, we isolated adult mouse ventricular myocytes (AMVMs) from hearts of mice following 1 day Ang II infusion. There was a 12-fold increase in MCP-1 mRNA in the AMVMs isolated from control Ang II-infused mice (Fig. 3.2B). Cardiomyocyte CaMKIIδ signaling was implicated because the increase in MCP-1 mRNA was significantly attenuated in myocytes isolated from CKO mice (Fig. 3.2B). AMVMs from untreated mice also responded to *in vitro* addition of Ang II (1 μmol/L) with CaMKIIδ-dependent increases in MCP-1 expression (Fig. A.6). This response was also attenuated by addition of BMS-345541, an inhibitor to I kappa B kinase the upstream kinase to nuclear factor kappa B, indicating a requirement for NFkB in

Ang II-induced inflammatory gene expression in cardiomyocytes (Fig. A.7). In addition, we showed by that cardiac MCP-1 protein was increased by Ang II infusion and that this response was inhibited in hearts lacking CaMKIIδ in the cardiomyocyte (Fig. 3.2C).

3.3.3 Angiotensin II-induced early inflammatory responses are not secondary to cell death or hypertension

Cell death can lead to release of factors that stimulate inflammation[59] raising the possibility that inflammation is diminished in the CKO mouse heart due to decreased CaMKIIδ-mediated cell death. Notably, however, we observed no increase in Evans blue dye penetration, TUNEL staining, or caspase 3 cleavage in 1 day Ang II-infused versus vehicle-infused mouse hearts (Fig. 3.4). Thus, neither significant necrotic nor apoptotic cell death are elicited in response to the short-term Ang II infusion that robustly increases inflammatory gene expression. We also tested possible differences in hypertensive effects of Ang II infusion in control and CKO mice. Ang II infusion increased blood pressure to a similar extent in both lines over 7 days with only a modest increase at 1 day of infusion (Fig. 3.5). These data support a transcriptional role of cardiomyocyte CaMKIIδ in initiating inflammation rather than effects of CaMKIIδ on cardiomyocyte viability or on systemic neurohumoral changes secondary to increased blood pressure.

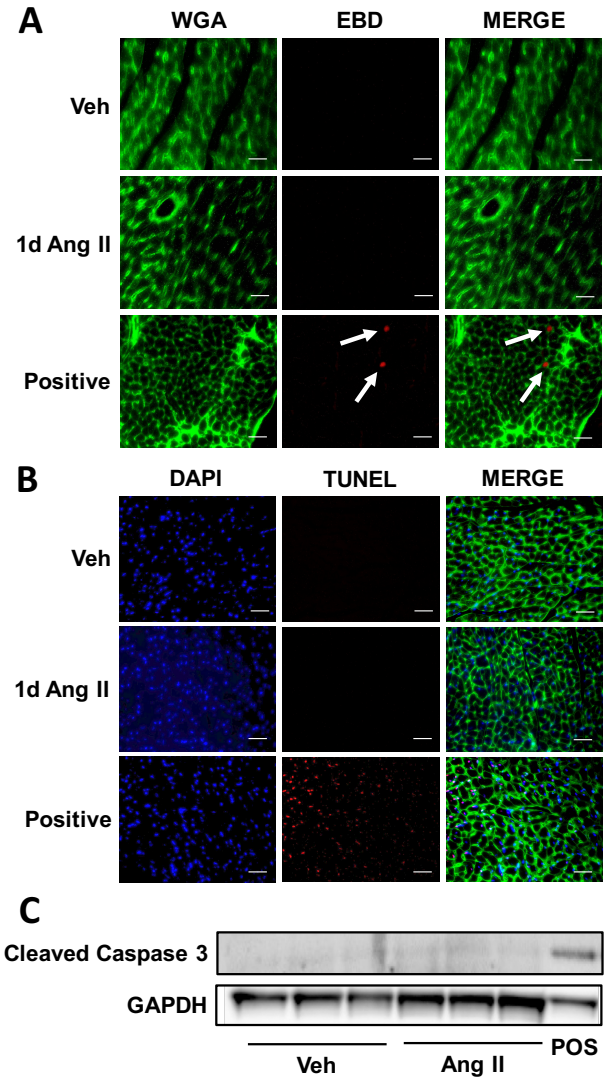

Figure 3.4. Angiotensin II infusion for 1 day does not cause cardiac cell death. **A,** Representative pictures of sections stained with wheat germ agglutinin (green) taken from control mice infused with Ang II for 1 day and injected with 100 mg/kg Evans blue dye (EBD) or vehicle (Veh). Positive control is 20 mg/kg doxorubicin injection 3 days before sacrifice. (n=6 each group) **B,** Representative pictures taken from control mice infused with Ang II for 1 day and stained using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Positive control is Ang II infusion for 7 days (n=6 each group). **C,** Western blot of cleaved caspase 3 protein in ventricular lysates of control mice infused with Ang II for 1 day. Positive control is neonatal rat ventricular myocytes treated with 5 μmol/L stuarosporine for 3 hours (n=3 each group). Scale bar = 50 μm.

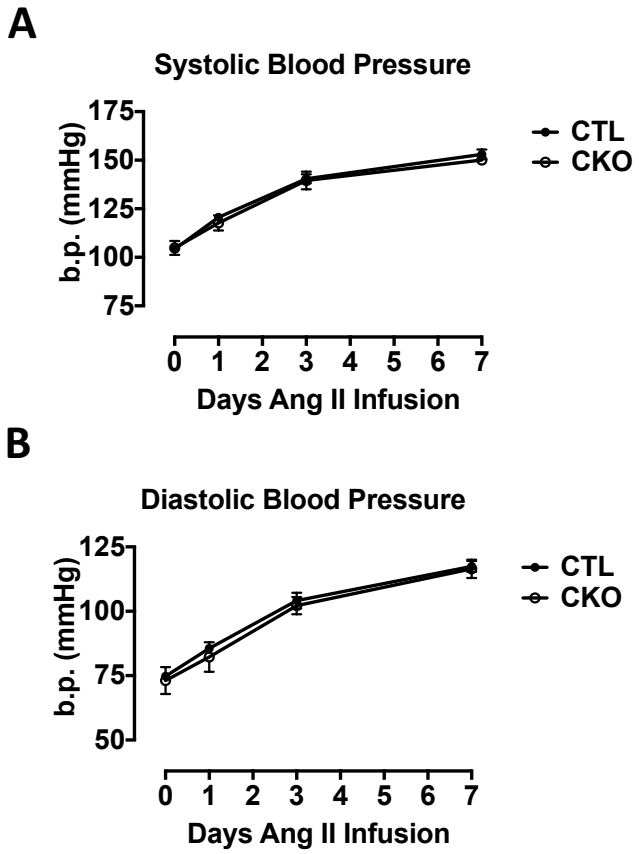

Figure 3.5. Angiotensin II infusion increases blood pressure to similar extents in control and cardiomyocyte-specific CaMKIIδ KO mice. Systolic (**A**) and diastolic (**B**) blood pressure measured in un-anesthetized mice at baseline, 1, 3, and 7 days Ang II infusion (n=10 each group).

3.3.4 Angiotensin II recruits macrophages to the heart through CaMKIIδ and MCP-1

MCP-1 recruits monocytes to inflamed tissues where they differentiate and become macrophages[134]. To determine if Ang II elicits CaMKIIδ-dependent accumulation of macrophages in the heart, we immunostained cardiac sections with the macrophage markers CD68 and F4/80. Both CD68 and F4/80 positive areas increased by

approximately 3-fold in control hearts after 1 day Ang II infusion. These increases were nearly fully abolished in hearts from cardiomyocyte-specific CaMKIIδ KO mice (Fig. 3.6A and 3.6B). To examine the role of MCP-1 in this response we treated control mice with RS102895, a highly selective small molecule antagonist of the monocyte C-C chemokine receptor type 2 (CCR2), the receptor for MCP-1[135]. Control mice were infused with Ang II as in previous experiments and injected with 10 mg/kg RS102895 or vehicle every 6 hours for 1 day, a dose and time course shown in the literature to achieve a plasma concentration sufficient to inhibit macrophage recruitment[135]. RS102895 treatment decreased Ang II-induced CD68 staining by more than 70% (Fig. 3.6C).

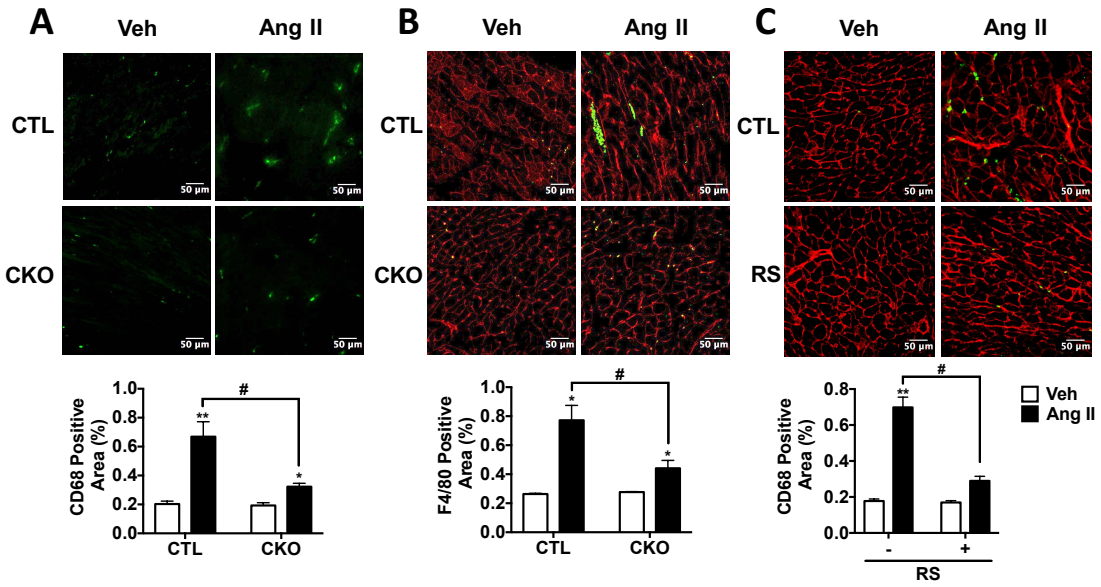

Figure 3.6. Ang II-induced macrophage accumulation in the heart is attenuated by CaMKIIδ gene deletion in the cardiomyocyte and MCP-1 receptor blockade. **A-C,** Representative pictures and quantitation of cardiac sections stained with antibodies to the macrophage markers CD68 (**A and C**) or F4/80 (**B**) taken from mice infused with Ang II for 1 day (n=3-6 each group). CD68 and F4/80 are green and wheat germ agglutinin is red. Mice in (**C**) were control mice infused with Ang II and injected with 10 mg/kg RS102895 (RS) or vehicle every 6 hours starting 1 hour prior to infusion. **P*<0.05 vs Veh, ***P*<0.01 vs Veh, #*P*<0.05 CTL vs CKO or Ang II alone vs Ang II + RS.

3.3.5 Angiotensin II activates the NLRP3 inflammasome through CaMKIIδ

Interleukin-1β mRNA was induced at 1 day Ang II infusion and this was the gene most highly dependent on cardiomyocyte CaMKIIδ (Fig. 3.2A). A 5-fold induction of IL-1β could be observed as early as 3 hours (Fig. 3.3). The IL-1β precursor protein is processed to its active form through the actions of caspase 1 in the inflammasome complex[126]. Expression of the key inflammasome component, NLRP3, like IL-1β, is

regulated through NFkB[126], suggesting that it might be regulated through CaMKIIδ. Indeed, control mice infused with Ang II for 1 day showed a 3-fold increase in cardiac NLRP3 protein expression and this response was significantly attenuated in mice lacking CaMKIIδ in the cardiomyocyte or injected with BMS to inhibit NFkB (Fig. 3.7A and B). The standard measure of inflammasome activation is an increase in caspase 1 activity[136]. One day Ang II infusion significantly increased caspase 1 activity as determined using a fluorometric activity assay (Fig. 3.7C). This response was significantly attenuated in hearts of CKO mice. The observed increases in NLRP3 and caspase 1 are evidence that Ang II regulates inflammasome assembly and activation in the heart and that cardiomyocyte CaMKIIδ mediates these responses.

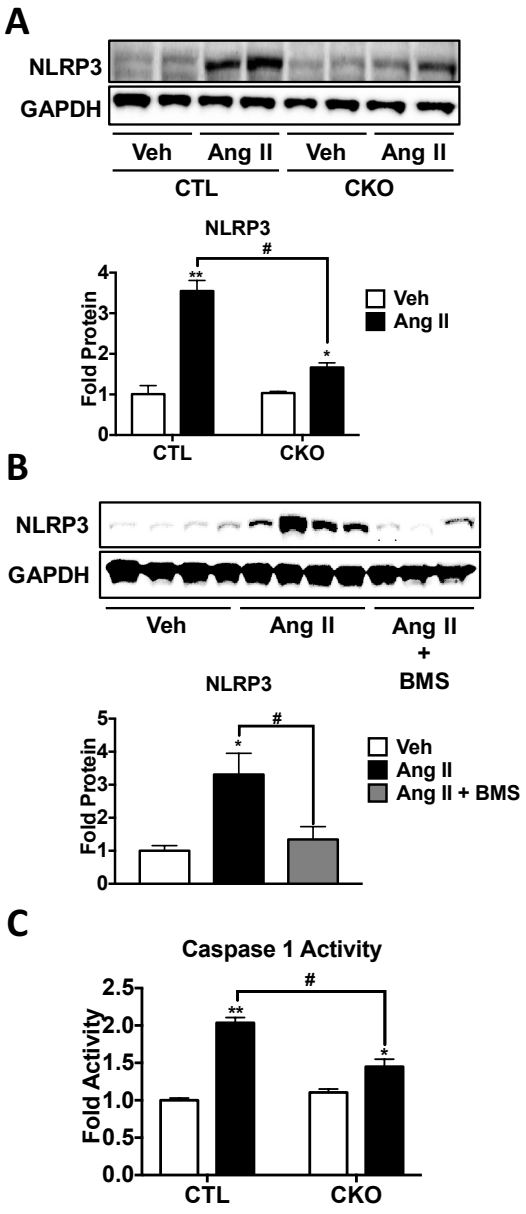

Figure 3.7. Ang II-induced priming and activation of the inflammasome is mediated through cardiomyocyte CaMKIIδ and NFkB. **A,** Western blot and quantitation of NLRP3 protein in ventricular lysates of control and CKO mice after 1 day Ang II infusion (n=4 each group). **B,** Western blot and quantitation of NLRP3 protein in ventricular lysates of control mice after 1 day Ang II infusion ± injection of BMS-345541 (BMS) (n=3-4 each group). **C,** Caspase 1 activity measured in ventricular lysates (n=4 each group). **P*<0.05 vs Veh, ***P*<0.01 vs Veh, #*P*<0.05 CTL vs CKO or Ang II vs Ang II + BMS.

3.3.6 Active CaMKIIδ regulates NLRP3 inflammasome priming and ROS-dependent inflammasome activation in cardiomyocytes

To directly show that CaMKIIδ can regulate the NLRP3 inflammasome in cardiomyocytes we infected neonatal rat ventricular myocytes (NRVMs) with control adenovirus expressing green fluorescent protein (AdGFP) or adenovirus expressing the constitutively active δ_C isoform of CaMKIIδ (AdCaMKIIδ). Expression of CaMKIIδ for 18 hours led to a robust increase in NLRP3 protein (Fig. 3.8A). CaMKIIδ has been demonstrated to promote generation of mitochondrial reactive oxygen species (ROS) in cardiomyocytes[36, 113, 137]. We determined that NRVMs infected with AdCaMKIIδ showed a robust increase in mitochondrial ROS as assessed by MitoSOX fluorescence (Fig. 3.8B). Since ROS are known activators of the inflammasome complex[138, 139] we extended our studies to determine whether CaMKIIδ regulates inflammasome activation through ROS. AdCaMKIIδ expression increased cardiomyocyte caspase 1 activity (Fig. 3.8C) and blocking ROS accumulation with the general or mitochondrial targeted ROS scavengers N-acetylcysteine or MitoTempo led to complete inhibition of this response (Fig. 3.8C).

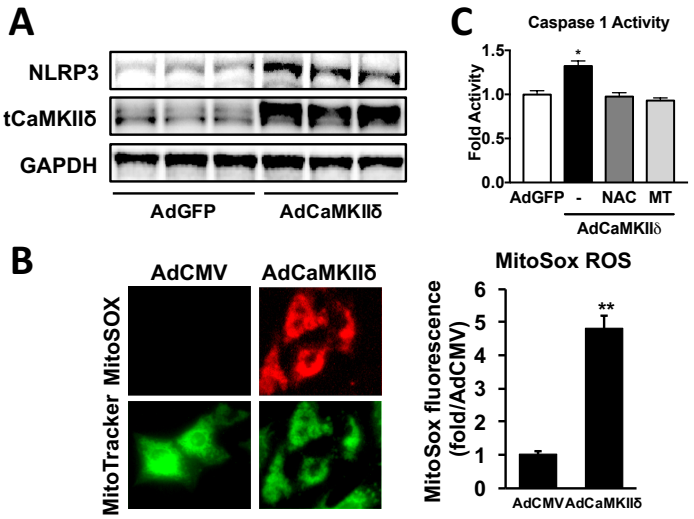

Figure 3.8. CaMKIIδ primes and activates the inflammasome in neonatal rat ventricular myocytes (NRVMs). **A,** Western blot of NLRP3 protein in NRVMs infected with adenovirus expressing GFP (AdGFP) or the active δ_C isoform of CaMKII (AdCaMKIIδ) (n=6 each group). **B,** Live cell images and quantitation of fluorescence in NRVMs infected with adenovirus expressing an empty CMV promotor (AdCMV) or AdCaMKIIδ. MitoSOX and MitoTracker green were loaded to visualize mitochondrial reactive oxygen species and mitochondria. (n=9 each group). **C,** Caspase 1 activity measured in NRVMs infected with AdGFP or AdCaMKIIδ ± pretreatment with 10 mmol/L N-acetylcysteine (NAC) or 10 μmol/L MitoTempo (MT) (n=9 each group). *P<0.05 vs AdGFP, **P<0.01 vs AdCMV.

3.3.7 Inhibition of the inflammasome attenuates Ang II-induced inflammation

IL-1β, a product of inflammasome activation, promotes and sustains pro-inflammatory cytokine expression and can contribute to recruitment of macrophages[140]. To test the role of inflammasome function in the induction of inflammatory genes and recruitment of macrophages in response to Ang II, mice were injected with MCC950,

an inhibitor of NLRP3 activation[141], beginning 1 hour prior to infusion with Ang II. MCC950 treatment significantly attenuated Ang II-induced MCP-1, MIP1α, CXCL1, CXCL2 and IL-6 mRNA expression (Fig. 3.9A) and led to nearly complete inhibition of the Ang II-induced accumulation of CD68 positive macrophages in the heart (Fig. 3.9B).

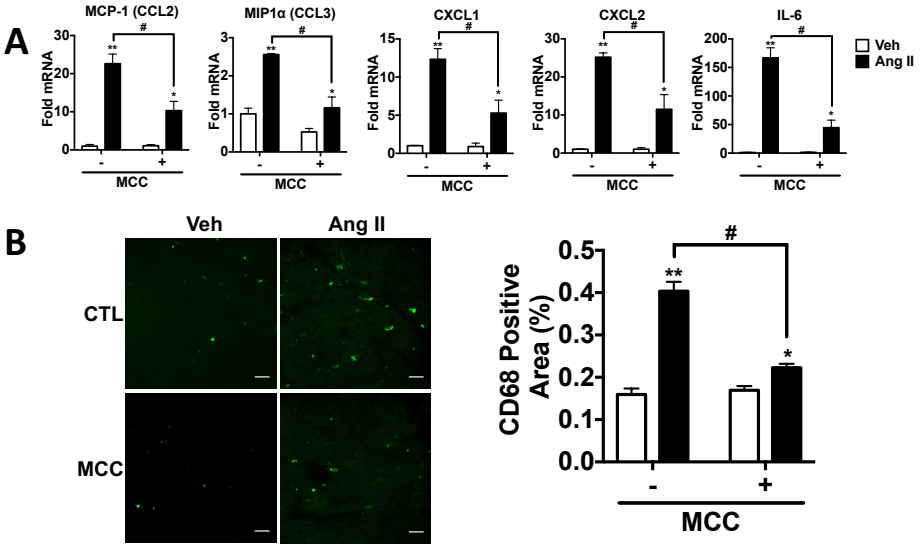

Figure 3.9. Inflammasome inhibition attenuates Ang II-induced inflammatory responses in the heart. **A,** mRNA expression of inflammatory chemokines and cytokines in ventricles of control mice infused with Ang II for 1 day and injected with 10 mg/kg MCC950 (MCC) every 12 hours starting 1 hour prior to infusion (n=4 each group). **B,** Representative pictures and quantitation of cardiac sections stained with antibody to CD68 (n=4 groups each). *P<0.05 vs Veh, **P<0.01 vs Veh, #P<0.05 Ang II alone vs Ang II + MCC. Scale bar = 50 μm.

3.3.8 Cardiomyocyte CaMKIIδ signaling contributes to angiotensin II-induced fibrosis

Ang II infusion leads to robust induction of myocardial fibrosis[38, 61, 63, 66, 142, 143]. The increase in fibrosis observed by Masson's trichrome

staining after 7 days of angiotensin II infusion was diminished by 60% in cardiomyocyte-specific CaMKIIδ KO mouse (Fig. 3.10A). The fibrotic gene markers collagen type 1α1 (Col1a1), collagen type 3α1 (Col3a1), and periostin were also upregulated in control Ang II-infused mouse hearts and these increases, evident at both 3 and 7 days, were significantly attenuated in hearts of mice lacking cardiomyocyte CaMKIIδ (Fig. 3.10B).

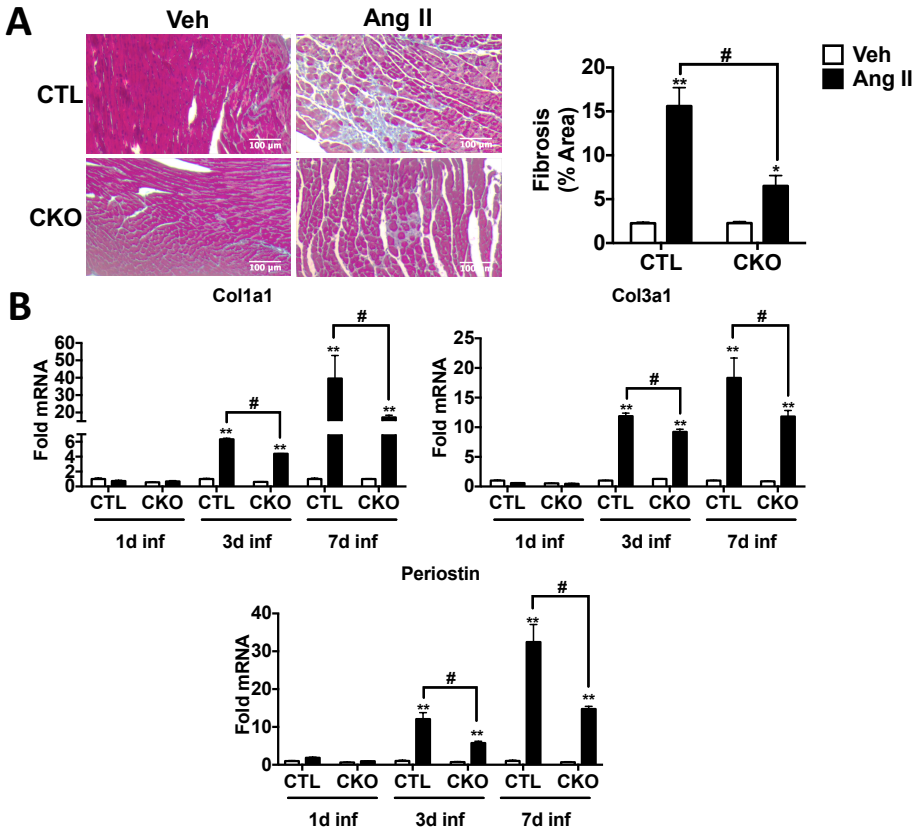

Figure 3.10. Cardiac fibrosis induced by 7 day Ang II infusion is attenuated in mice lacking CaMKIIδ in the cardiomyocyte. **A,** Representative pictures and quantitation of sections stained with Masson's trichrome (n=3 each group). **B,** mRNA expression of fibrotic markers collagen 1α1 (Col1a1), collagen 3α1 (Col3a1), and periostin in ventricles as measured by qPCR (n=3-6 each group). *P<0.05 vs Veh, **P<0.01 vs Veh, #P<0.05 CTL vs CKO.

Expression of mRNA for pro-fibrotic mediators including CCN family member 1/cysteine-rich angiogenic factor 61 (CCN1), CCN family member 2/connective tissue growth factor (CCN2), and transforming growth factor β (TGFβ), was also increased after 7 days of Ang II infusion and attenuated in the cardiomyocyte CaMKIIδ KO (3.11).

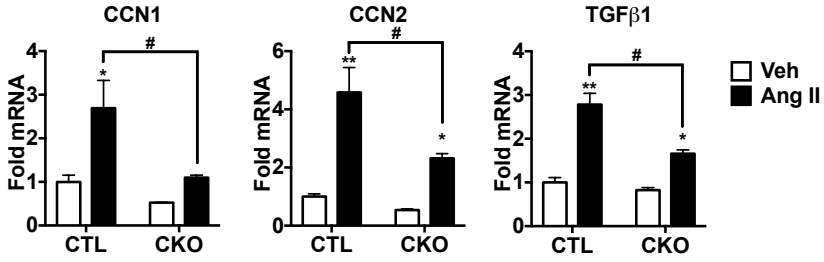

Figure 3.11. Angiotensin II induces profibrotic mediator expression through CaMKIIδ signaling in the cardiomyocyte. mRNA expression of profibrotic mediators in ventricular lysates of control and CKO mouse hearts after 7 days of Ang II infusion (n=3 each). *P<0.05 vs Veh, **P<0.01 vs Veh, #P<0.05 CTL vs CKO.

3.3.9 MCP-1 and the inflammasome are involved in angiotensin II-induced fibrosis

There is considerable evidence that inflammation plays a role in development of fibrosis[38-41]. Accordingly, we asked whether interventions that attenuated Ang II-induced inflammation also diminished fibrosis. We tested both the MCP-1 and NLRP3 inflammasome inhibitors for their effects on the fibrotic responses seen at 3 days Ang II infusion. Treatment with RS102895 decreased the area of Ang II-induced fibrosis by 40% and

significantly attenuated Col1a1, Col3a1, and periostin expression (Fig. 3.12A and 3.12B). Treatment with MCC950 likewise attenuated Ang II-induced fibrosis (Fig. 3.12C) and significantly attenuated expression of the fibrotic gene markers Col1a1, Col3a1, and periostin (Fig. 3.12D). Thus both MCP-1 signaling and inflammasome activation are dependent on cardiomyocyte CaMKIIδ and contribute to Ang II-induced fibrosis.

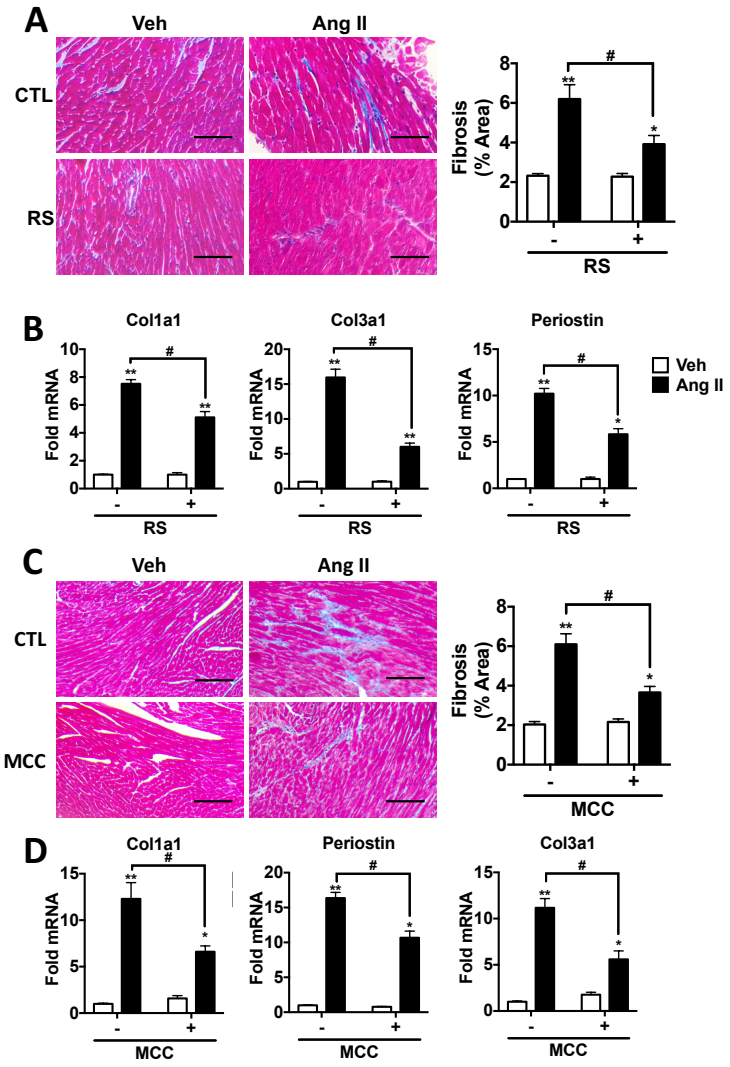

Figure 3.12. Inhibition of MCP-1 or inflammasome signaling attenuates cardiac fibrosis induced by Ang II infusion. Control mice were injected with either 10 mg/kg RS102895 every 6 hours (**A and B**) or 10 mg/kg MCC950 (MCC) every 12 hours (**C and D**) starting 1 hour prior to a 3-day Ang II infusion. **A,** Representative pictures and quantitation of sections stained with Masson's trichrome (n=6 each group). **B,** mRNA expression of fibrotic markers collagen 1α1 (Col1a1), collagen 3α1 (Col3a1), and periostin in ventricles as measured by qPCR (n=6 each group). **C,** Representative pictures of sections stained with Masson's trichrome (n=6-12 each group). **D,** mRNA expression of fibrotic markers collagen 1α1 (Col1a1), collagen 3α1 (Col3a1), and periostin in ventricles as measured by qPCR (n=6-12 each group). *P<0.05 vs Veh, **P<0.01 vs Veh, #P<0.05 Ang II alone vs RS/MCC. Scale bar = 100 μm.

3.4 Discussion

Cardiac inflammation, a hallmark of heart failure, is a process that is commonly associated with the necrotic cell death that accompanies myocardial infarction or ischemia/reperfusion injury[144-147]. Here we address the question of how rapid cardiac inflammatory responses are initiated by Ang II infusion, in the absence of acute loss of cardiomyocytes. Elucidating the molecular mechanisms leading to inflammation is critical to defining pathways that could prevent adverse remodeling and heart failure development.

The studies reported here are the first to demonstrate that there is a critical role for cardiomyocytes, and for CaMKIIδ activated in this compartment, in Ang II-induced inflammatory gene expression and inflammasome activation. These responses serve, in turn, to recruit macrophages and further enhance and sustain inflammatory gene expression. There are a growing number of reports demonstrating that "cardiokines" are generated in various heart cells including fibroblasts, endothelial cells, myocytes and resident macrophages[148-150]. The cardiomyocyte is not, however, appreciated as an essential generator of inflammatory mediators (except due to its necrotic cell death). The work reported here identifies the cardiomyocyte as a site for initiation of inflammatory chemokine and cytokine mRNA expression and

subsequent signaling, as evidenced by markedly attenuated inflammatory gene expression and inflammatory cell accumulation in hearts lacking CaMKIIδ in the cardiomyocyte. MCP-1, shown in previous studies using Ang II infusion to be required for cardiac inflammation and subsequent fibrosis[66], is at least one of the critical pro-inflammatory chemokines that is increased in cardiomyocytes through the actions of CaMKIIδ.

Recent evidence has implicated the inflammasome in the inflammatory response to myocardial infarction and ischemia/reperfusion[129, 130], but a role for the inflammasome in cardiac responses to Ang II has not been demonstrated. We show that infusion of Ang II increases IL-1β mRNA and NLRP3 protein, indicative of inflammasome priming and leads to activation of the inflammasome as evidenced by increases in caspase 1 enzymatic activity in the mouse heart. Remarkably CaMKIIδ is a key mediator of this process as both responses are attenuated by cardiac-specific deletion of CaMKIIδ. Several reports implicate cardiac fibroblasts as the major source of inflammasome signaling in the heart and suggest that cardiomyocytes have a limited role in inflammasome signaling[129, 151, 152]. However, other studies have used immunofluorescence staining to identify the cardiomyocyte as a site of inflammasome signaling[130, 153, 154]. Whether

inflammasome priming and activation in response to Ang II infusion occurs within cardiomyocytes or involves signaling to fibroblasts or other cells remains to be determined. In either case, we demonstrate here that expression of constitutively active CaMKIIδ in myocytes can increase NLRP3 protein and caspase 1 activity and that CaMKIIδ signaling in cardiomyocytes is a necessary component of inflammasome priming and activation by Ang II.

Necrotic cell death induces inflammasome activation and subsequent inflammation through release of damage-associated molecular patterns[155]. However, we observe inflammasome activation, CaMKIIδ-mediated inflammatory gene expression, and macrophage accumulation within 1 day of Ang II treatment, a time point shown here and in the literature to not be associated with death of cardiac myocytes[156]. Thus, while CaMKIIδ has been implicated in processes that elicit various forms of cardiomyocyte cell death[29-33], the physiological levels of CaMKIIδ activation achieved at short times of Ang II infusion appear to directly signal through a rapidly activated cardiomyocyte autonomous transcriptional pathway rather than through factors released by dying cells. We demonstrate here that Ang II infusion activates the transcription factor NFkB in control but not in CKO mouse hearts. Furthermore, we show through use of inhibitors that blockade of

NFkB activation is sufficient to attenuate increases in NLRP3 protein induced by Ang II. Activated NFkB expressed in cardiomyocytes has been shown to induce cardiac inflammation[157]. Thus activation of NFkB provides a mechanism for stimuli that elicit CaMKIIδ signaling to induce inflammatory genes as well as inflammasome priming (upregulation of IL-1β and NLRP3).

Previous reports have implicated CaMKIIδ in development of cardiac fibrosis induced by pressure overload, Gαq overexpression, sustained β-adrenergic stimulation, and myocardial infarction[20, 22, 35-37], but the mechanism by which CaMKIIδ contributes to cardiac fibrosis has not been determined. We show here and in previous studies that CaMKIIδ does not play a role in hypertrophy induced by Ang II infusion (Fig. A.8), pressure overload, Gαq overexpression, and sustained β-adrenergic stimulation. Thus, CaMKIIδ does not appear to drive fibrosis through effects on hypertrophy. The findings presented here demonstrate that genetic deletion of CaMKIIδ not only ameliorates fibrosis but markedly inhibits expression of the inflammatory gene MCP-1 and activation of the inflammasome, processes that contribute to macrophage recruitment. These data are consistent with there being a prominent role for CaMKIIδ-mediated inflammatory cell recruitment in Ang II-induced fibrosis. It should be noted, however, that Ang II also induces CCN1, CCN2, and

TGFβ, proteins that could act directly on fibroblasts to induce fibrosis. While it is not clear whether the mRNA for these profibrotic proteins is upregulated in cardiomyocytes, fibroblasts or other cardiac cells, what is noteworthy is that these changes are also secondary to the actions of Ang II on cardiomyocyte CaMKIIδ signaling. We have investigated the role of CCN1 expressed in the cardiomyocyte in the Ang II-induced fibrotic response. Surprisingly, Ang II-induced fibrosis was potentiated by deletion of CCN1 in the cardiomyocyte (Fig. A.9).

Angiotensin II signaling is an evolutionarily conserved neurohormonal pathway that can elicit myriad pathophysiological effects on multiple tissues and cell types but the cardiomyocyte is not considered to be an essential site at which Ang II acts to initiate cardiac inflammation. Here we show that Ang II infusion activates NFkB, rapidly regulates MCP-1 expression and triggers subsequent cardiac macrophage recruitment to the heart through cardiomyocyte CaMKIIδ signaling. CaMKIIδ activation in cardiomyocytes by Ang II infusion also leads to priming and activation of the inflammasome. Cardiomyocyte CaMKIIδ, MCP-1 and the inflammasome are all implicated, through gene deletion or inhibitor studies, in Ang II-induced macrophage recruitment and fibrosis. This study reveals an unexpected central role for CaMKIIδ signaling in the cardiomyocyte to inflammatory responses, immune cell

recruitment, and cardiac fibrosis. We are currently extending this work to examine the role of CaMKIIδ-mediated inflammation in development of adverse remodeling and dysfunction in pressure overload-induced heart failure. Treatment of cardiac inflammation through pharmacological inhibition of CaMKIIδ or its immediate downstream effectors may provide significant benefits in preventing heart failure progression.

3.5 Experimental Procedures

3.5.1 Animals

Black Swiss mice in which CaMKIIδ was genetically deleted in the cardiomyocyte (cardiomyocyte-specific CaMKIIδ KO mice) were generated by crossing homozygous floxed CaMKIIδ (CaMKIIδ$^{fl/fl}$) mice with MLC2v-Cre mice as described. There were no baseline changes in ventricular structure or function as compared to wild type (WT) and CaMKIIδ$^{fl/fl}$ (control) mice[11]. Male control and cardiomyocyte-specific CaMKIIδ KO mice 8-12 weeks of age were infused with 1.5 μg/kg/min angiotensin II (Ang II) or vehicle (saline) using unprimed Alzet model 1007D micro-osmotic pumps for up to 7 days. There were no differences in inflammatory responses to Ang II among wildtype, CaMKIIδ$^{fl/fl}$ and the unfloxed MLC2v-Cre mice (data not shown). Accordingly, we used CaMKIIδ$^{fl/fl}$ mice as controls for this study. In some experiments, mice were also injected every 6 hours with 10 mg/kg RS102895 (Tocris), every 12

hours with 10 mg/kg MCC950 (Millipore), or vehicle (1:1 water:DMSO or saline) for up to 3 days. Mice were also injected with BMS-345541 or vehicle (water) at 3 time points throughout a 1-day Ang II infusion, a regimen designed using published pharmacokinetic parameters[158]. Doses and injection times included 30 mg/kg at T-0 hrs of Ang II infusion, 15 mg/kg at T-5 hrs, and 60 mg/kg at T-8 hrs. Animal studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of California – San Diego.

3.5.2 CaMKII activity assay

CaMKII activity was measured using previously published protocols[10, 35]. In brief, hearts were isolated, ventricles homogenized in lysis buffer, and immediately assayed in assay buffer containing 50 mmol/L HEPES, 10 mmol/L magnesium acetate, 1 mg/ml BSA, 20 μmol/L Syntide-2 (a synthetic CaMKII-specific peptide substrate), 1mmol/L DTT, 400 nmol/L [γ-^{32}P]ATP, pH 7.5, and 1 mmol/L EGTA (for measurement of autonomous activity) or 500 μmol/L $CaCl_2$ and 1 μmol/L calmodulin (for measurement of maximal activity). The reaction was carried out at 37°C for 10 min and blotted onto Whatman P81 phosphocellulose paper. Percent activation was calculated as the ratio of autonomous activity to maximal activity.

3.5.3 Nuclear fractionation

Ventricular tissue was fractionated using previously published protocols[10, 159]. In brief, frozen ventricular tissue was homogenized in lysis buffer containing 70 mmol/L sucrose, 190 mmol/L mannitol, 20 mmol/L HEPES, and 200 μmol/L EDTA using a dounce tissue grinder. Lysates were centrifuged at 600g for 10 min at 4°C. Supernatants were aspirated and pellets washed three times by resuspending pellets with lysis buffer and centrifuging at 600g for 10 min at 4°C. Supernatants were aspirated, pellets resuspended in nuclear extraction buffer containing 20 mmol/L HEPES, 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L magnesium chloride, and 200 μmol/L EDTA and incubated on ice for 10 min. Nuclear lysates were then centrifuged at 600g for 10 min at 4°C and supernatants collected (nuclear fraction).

3.5.4 Adult mouse ventricular myocyte isolation

Adult mouse ventricular myocytes were isolated from male control and cardiomyocyte-specific CaMKIIδ KO mice using a modified published protocol[160]. In brief, mice were anesthetized with ketamine/xylazine, hearts removed, cannulated, and perfused at 37 °C at a rate of 3 ml/min. Initially, hearts were perfused for 3 min with a calcium-free buffer. Hearts were then digested with buffer containing 12.5 μmol/L calcium and 0.34 mg/ml Liberase TH (Roche) for 7-10 mins.

Hearts were removed from the cannula, atria and vessels removed, and ventricles manually dissociated using micro forceps and by multiple passages through a Pasteur pipette. Cells were strained through a 100 μm strainer and the Liberase TH was deactivated by resuspending the cells in perfusion buffer containing 10% fetal bovine serum (STOP buffer). Cells were pelleted by gravity sedimentation, supernatant aspirated, and cells washed with STOP buffer two times. After the final wash, supernatant was aspirated and pelleted cells immediately lysed for RNA extraction.

3.5.5 Histological Analyses

Hearts were fixed in 4% PFA for 1 day, cryoprotected in 30% sucrose, frozen in O.C.T. compound (Fisher), and cryosectioned (5 μm). Sections were stained with 1:100 F4/80 (Serotec, MCA497) or 1:100 CD68 (Abcam, ab53444) and visualized with 1:750 Alexa Fluor 488 secondary antibody (Life Technologies) on a Zeiss Axio Observer Z1, inverted microscope. Images (20x magnification) were quantified as percentage area using ImageJ software (version 1.48g, NIH, Bethesda, MD, USA). Paraffin embedded sections (5 μm) were used to measure fibrosis and cell death. Sections were stained with Masson's trichrome to visualize fibrosis. Percentage area fibrosis was calculated from greater than 15

randomly acquired images per cardiac section (20x) using ImageJ software.

3.5.6 Cell Death

Necrotic cells were examined by intraperitoneally injecting mice with 100 mg/kg Evan's blue dye (Sigma Aldrich), which accumulates in necrotic cells and auto-fluoresces red, 18 hours prior to sacrifice[32, 161]. Apoptotic cells were examined using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche). Western blotting for cleaved caspase 3 (CST, 9661) in ventricular lysates was used as a second measure of apoptotic cell death.

3.5.7 RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was isolated from ventricles and adult mouse ventricular myocytes using TRIzol reagent as described by the manufacturer (ThermoFisher). Single stranded cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression of CCL2, CCL3, CXCL1, CXCL2, IL-1β, IL-6, Col1a1, Col3a1, periostin, TGFb, CCN1, and CCN2 were measured using a pre-validated Taqman® Gene expression assay (Integrated DNA Technologies) on a Fast 7500 qPCR machine (Applied Biosystems).

3.5.8 Caspase 1 activity assay

Caspase 1 activity was measured in ventricular and neonatal rat

ventricular myocyte lysates using a pre-validated fluorometric caspase 1 activity kit as described by the manufacturer (Abcam, ab39412).

3.5.9 Neonatal rat ventricular myocyte isolation, culture, and infection

Hearts were isolated from 1-2 day old Sprague-Dawley rat pups, digested in collagenase II (Worthington), and myocytes purified by passage through a percoll gradient. Myocytes were plated at a density of $3.5x10^4$ cells/cm^2 overnight at 37°C in Dulbecco's Modified Eagle Medium supplemented with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, and 100 ug/ml streptomycin. After overnight plating, cells were washed with serum free medium and starved for 5 hours. Adenovirus expressing the constitutively active δ_C isoform of CaMKIIδ (AdCaMKIIδ) or GFP (AdGFP) was applied to cultures for 3 hours at a multiplicity of infection (MOI) of 100. Cultures were washed with serum free medium and incubated overnight before processing for Western blotting or caspase 1 activity. To inhibit ROS formation, 10 mmol/L N-acetylcysteine (Sigma) or 10 μmol/L MitoTempo (Enzo Life Sciences) was added to cultures 1 hour prior to infection with adenovirus. To visualize ROS generation, live cells infected with AdCaMKIIδ or adenovirus containing an empty CMV promoter (AdCMV) at an MOI of 100 were loaded with MitoSOX (5 μmol/L) or MitoTracker green (1 mmol/L) and fluorescence measured using a Zeiss Axio Observer Z1, inverted

microscope.

3.5.10 Immunoblotting

Western blot analysis was performed on snap-frozen ventricular tissue. Tissue samples were lysed in radio-immunoprecipitation assay buffer and protein content measured via a bicinchoninic acid assay (ThermoFisher). Equal amounts of cardiac protein (30 μg) were heated in lithium dodecyl-sulfate with 100 mM dithiothreitol at 70 °C for 10 min. Proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Blots were blocked in 5% milk 1x tris-buffered saline/tween 20 (TBST) before incubation overnight at 4 °C with 1:250 cleaved caspase 3 (CST, 9661), 1:1000 GAPDH (CST, 2118), 1:1000 lamin a/c (CST, 2032), 1:500 MCP-1 (CST, 2029), 1:500 NLRP3 (CST, 15101), 1:1000 P65 (CST, 8242), 1:1000 RhoGDI (CST, 2564), and 1:5000 CaMKIIδ (Don Bers Laboratory). Blots were washed with 1x TBST, incubated with HRP-conjugated secondary antibody (Sigma Aldrich) at room temperature for 1 hour, washed with 1x TBST, and visualized by chemiluminescence (SuperSignal West Femto, ThermoFisher).

3.5.11 Statistical analyses

All results are presented as mean ± SEM and groups were compared using t-test, one-way, or two-way ANOVA. All statistics were

calculated using GraphPad Prism 7. P values of <0.05 were considered significant.

3.6 Acknowledgements

Chapter 3 will be submitted for publication upon completion of follow-up studies. The dissertation author was the primary author of this work in collaboration with Takeshi Suetomi, Audrey Nickle, Shigeki Miyamoto, and Joan Heller Brown. This work was supported by the National Institutes of Health (NIH) grants P01HL080101, R01HL105242, and R37HL028143 to J.H. Brown and NIH grant R56HL097037 and American Heart Association grant 15GRNTZ297009 to S. Miyamoto. A. Willeford was supported by NIH Research Training Grants T32 GM007752 and T32DK007541. T. Suetomi was supported by the Uehara Memorial Foundation (Japan). A. Nickle was supported by the UC San Diego Department of Pharmacology Summer Undergraduate Research Fellowship. We thank Jeff Smith for his assistance in adenoviral synthesis and amplification, Melissa Barlow for animal husbandry, and Valerie Tan for neonatal cell isolations.

Chapter 4

Conclusion and Future Directions

4.1 Summary

The calcium/calmodulin-dependent protein kinase II δ (CaMKIIδ) signaling pathway serves an essential role in development of cardiac arrhythmia, structural remodeling, and heart failure[5, 6, 21, 23, 24, 105]. Our work focused on the requirement for CaMKIIδ in maladaptive cardiac responses induced by sustained β-adrenergic receptor stimulation and angiotensin II (Ang II) infusion, both of which are known to activate CaMKIIδ and contribute to heart failure development[19, 25, 72, 79].

Our work showed for the first time that CaMKIIδ mediates cardiac remodeling and decompensation in response to chronic β-adrenergic receptor stimulation. Deletion of CaMKIIδ attenuated chronic isoproterenol-induced sarcoplasmic reticulum calcium leak, cardiac

fibrosis, and heart failure development while hypertrophic and acute inotropic and chronotropic responses to isoproterenol (ISO) were preserved. CaMKIIδ appeared to act through increasing sarcoplasmic reticulum calcium leak to promote heart failure development since this response was attenuated by inactivation of the CaMKIIδ-specific phosphorylation site on RyR2.

The body of my thesis research examined the role of CaMKIIδ in the cardiomyocyte in inflammatory gene expression and cell recruitment as well as fibrosis induced by a non-injury/cell death model. In these studies, we use Ang II infusion, a common non-ischemic hypertensive stressor, and cardiomyocyte-specific CaMKIIδ KO mice. Ang II infusion stimulated NFkB activation and rapidly regulated MCP-1 expression and macrophage recruitment to the heart through cardiomyocyte CaMKIIδ. The inflammasome was also primed and activated by Ang II through CaMKIIδ signaling from the cardiomyocyte. Priming of the inflammasome was dependent on NFkB activation, suggesting a pathway by which CaMKIIδ activated by Ang II can mediate inflammasome priming. Inflammatory signals mediated by CaMKIIδ were also suggested to be linked to Ang II-induced fibrosis since CaMKIIδ, MCP-1, and the inflammasome were all demonstrated to be essential in the development of fibrosis. This study showed for the first time that CaMKIIδ

mediates inflammatory gene expression and cell recruitment through a cell death-independent, cardiomyocyte autonomous transcriptional pathway involving MCP-1 and the inflammasome leading to fibrosis.

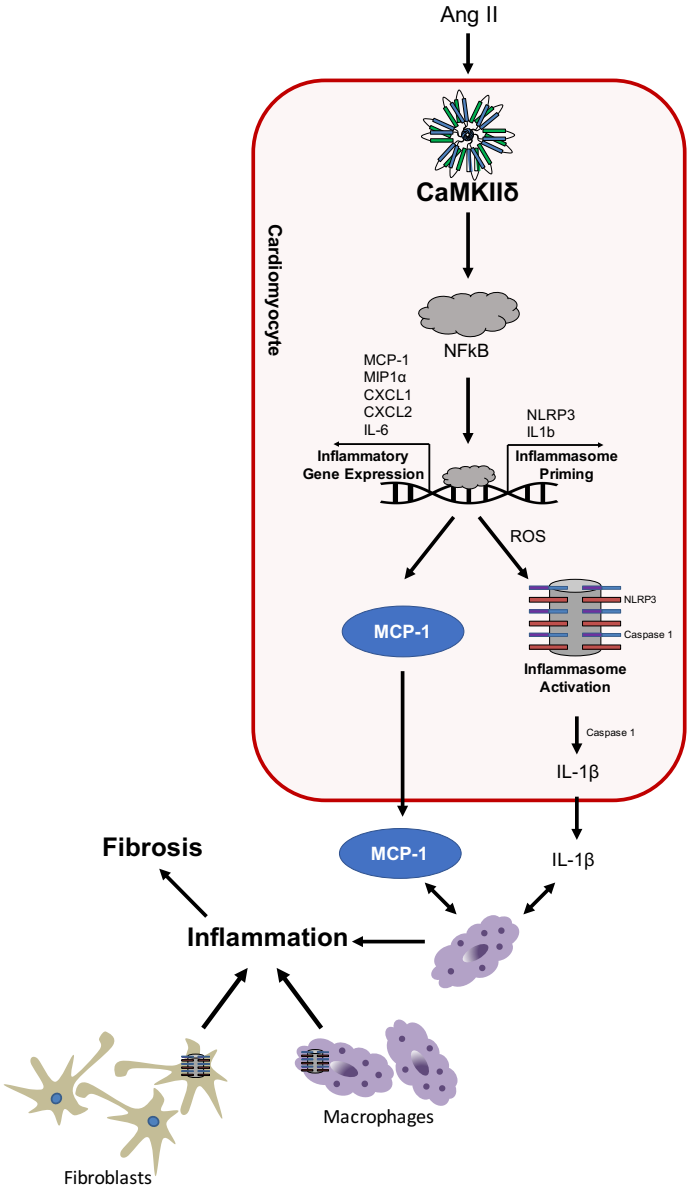

Figure 4.1. Proposed pathway by which cardiomyocyte CaMKIIδ initiates Ang II induced inflammation and fibrosis.

4.2 Future Studies

4.2.1 Cellular location of inflammasome signaling in Ang II-induced inflammatory responses and contribution of interleukin 1β

We have demonstrated that cardiomyocyte CaMKIIδ signaling plays a critical role in inflammasome priming and activation in the heart but are interested in determining the site at which the inflammasome is activated by CaMKIIδ signaling from the myocyte. We will infuse mice with Ang II for 1 day, isolate the cardiomyocyte and non-cardiomyocyte compartments and then carry out Western blotting for NLRP3 protein and measure caspase 1 activity in these compartments. If CaMKIIδ signals to the inflammasome within the myocyte we expect to see increases in NLRP3 and IL-1β mRNA, indicative of priming, and as well as increases in caspase 1 activity, indicative of activation of the inflammasome in isolated cardiomyocytes. We will also determine whether IL-1β, which is cleaved by activated caspase 1, is necessary for Ang II-induced inflammatory responses and fibrosis. This experiment parallels what was done with the inhibitor to the receptor to MCP-1 (RS-102895). Specifically, we will treat Ang II-infused mice with anakinra, an interleukin 1 receptor antagonist (provided by Dr. Hal Hoffman), and measure inflammatory gene expression, inflammatory cell recruitment, and fibrosis in the heart. We expect blockade of the IL-1 receptor to attenuate Ang II-induced

inflammatory responses and fibrosis. Additivity with RS-102895 will also be tested to determine if these are parallel or sequential pathways.

4.2.2 Differential pro-inflammatory signaling by CaMKIIδ splice variants

CaMKIIδ is expressed as multiple splice variants, the predominant two in the heart being CaMKIIδ_B and CaMKIIδ_C[4]. The δ_C splice variant has been demonstrated to be preferentially upregulated in response to TAC and to be a driver of deleterious signaling in the heart[46]. In contrast, CaMKIIδ_B appears to have a protective role since increased nuclear localization of the splice variant is associated with enhanced protective gene expression and cardiomyocyte survival[44, 45]. Preliminary data from our laboratory demonstrate that transgenic mice in which CaMKIIδ_C is re-expressed in a knockout background show increased expression of pro-inflammatory genes in the heart, over a time course that parallels development of heart failure in these mice. On the other hand, hearts of CaMKIIδ_B transgenic mice show no induction of these same genes (Fig. A.10). These preliminary data together with published studies showing selective activation of NFkB by active CaMKIIδ_C[10] suggest that it is the CaMKIIδ_C subtype, activated in the cardiomyocyte compartment, that initiates inflammation and subsequent cardiac remodeling and heart failure development. We have generated adenovirus expressing the CaMKIIδ_B or CaMKIIδ_C subtype which will be injected into CaMKIIδ KO

mice 2 weeks prior to Ang II treatment (a time we have shown to be sufficient to increase protein in the heart). We will then determine whether the ability of Ang II to trigger inflammatory gene expression, inflammatory cell recruitment, and contribute to fibrosis is restored by either or both subtypes.

4.2.3 Contribution of monocyte chemotactic protein-1 expression in cardiomyocytes to Ang II-induced inflammatory responses

Monocyte chemotactic protein-1 (MCP-1) is a pro-inflammatory chemokine shown to mediate inflammation and fibrosis induced by Ang II[66, 80]. We show in our studies that MCP-1 is regulated by CaMKIIδ and expressed in cardiomyocytes. To directly demonstrate that MCP-1 release from cardiomyocytes contributes to Ang II-induced inflammatory gene expression and cell recruitment, we have generated cardiomyocyte-specific MCP-1 knockout mice which will be infused with Ang II. These mice will further be used to relate inflammasome signaling and fibrosis induced by Ang II to MCP-1 signaling from the cardiomyocyte. We expect MCP-1 gene deletion in the cardiomyocyte to attenuate Ang II-induced inflammatory gene expression, inflammatory cell recruitment, inflammasome priming/activation, and fibrosis in the heart. We will also cross these mice with the CaMKIIδ$_C$ transgenic to determine whether blockade of MCP-1 signaling from the

cardiomyocyte is sufficient to reverse the spontaneous inflammatory response and heart failure development induced by active CaMKIIδC.

4.2.4 Role of CaMKIIδ-mediated inflammatory signaling in development of adverse remodeling and dysfunction in heart failure

Pressure overload has been demonstrated to induce cardiac inflammation[62, 67-69, 71] and we have shown in published studies that CaMKIIδ is robustly activated by pressure overload and mediates the transition from hypertrophy to heart failure[22, 46]. We predict that this occurs because CaMKIIδ, activated in cardiomyocytes by pressure overload, initiates inflammation and remodeling. We will also incorporate the use of MCP-1 knockout mice in these studies to determine the extent to which MCP-1 signaling from the myocyte is required in pressure overload-induced heart failure.

4.3 Concluding Remarks

The major studies of my dissertation reveal previously undescribed pathways by which CaMKIIδ mediates pathophysiological cardiac responses. We demonstrate a cell death-independent cardiomyocyte autonomous transcriptional role for CaMKIIδ in initiating inflammatory gene expression and cell recruitment. We further show that CaMKIIδ signaling in the cardiomyocyte regulates MCP-1 expression and the inflammasome, both of which are involved in inflammatory responses

and fibrosis induced by Ang II infusion. These studies are important as they offer new paradigms for future studies of cardiomyocytes and CaMKIIδ in inflammation. While inhibition of inflammation has met with limited success, blocking the activity of factors that trigger inflammation, such as CaMKIIδ, may provide significant benefit in treatment of heart failure.

Appendix

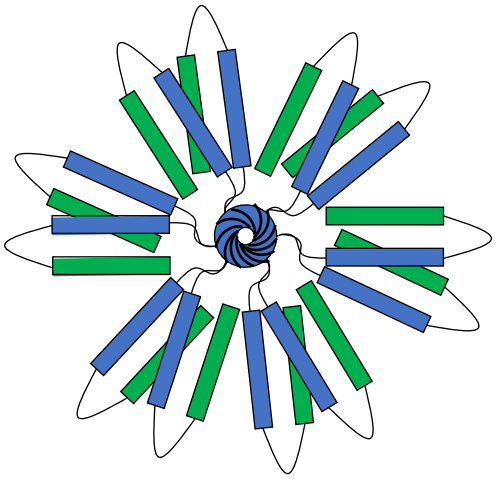

Figure A.1. General structure of the multimeric holoenzyme CaMKII

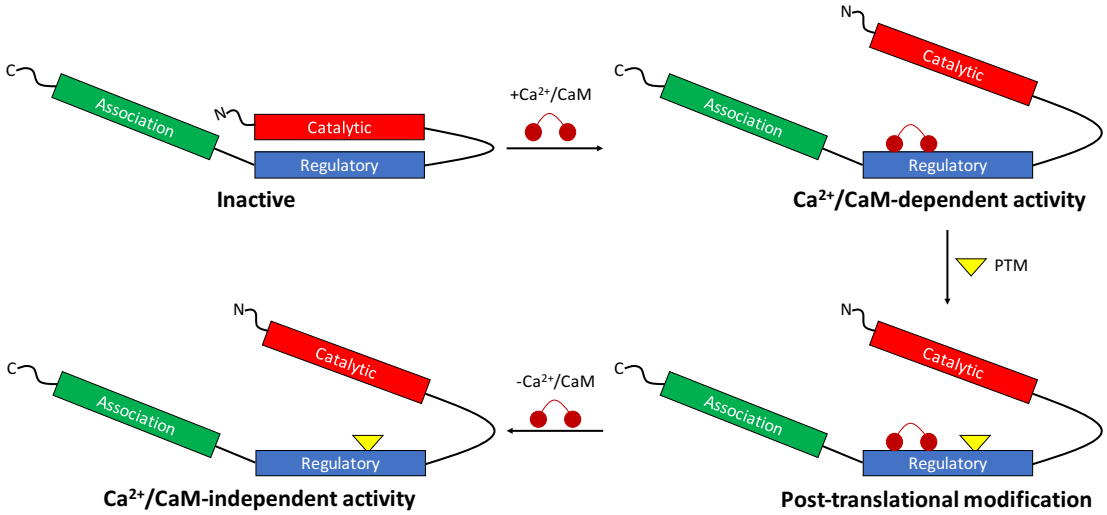

Figure A.2. Mechanism of CaMKII activation by post-translational modification (PTM).

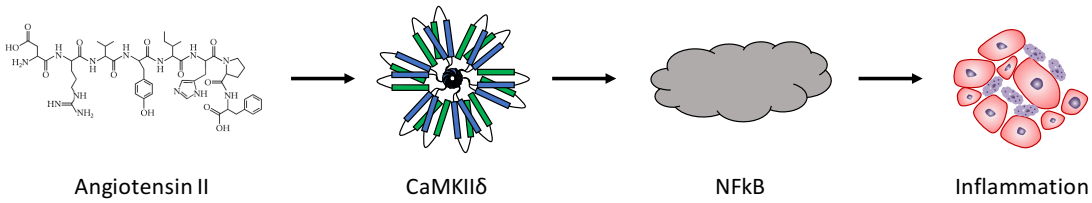

Figure A.3. Hypothesis of CaMKIIδ-mediated inflammation in response to a non-ischemic stress i.e. angiotensin II.

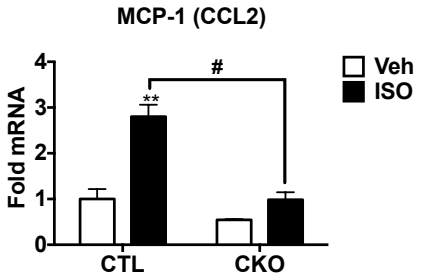

Figure A.4. Isoproterenol (ISO) infusion for 1 week induces monocyte chemotactic protein 1 (MCP-1) expression in the heart and requires CaMKIIδ. MCP-1 mRNA in whole ventricular lysates as measured by qPCR (n=3 each group). **P<0.01 vs Veh, #P<0.05 CTL vs CKO.

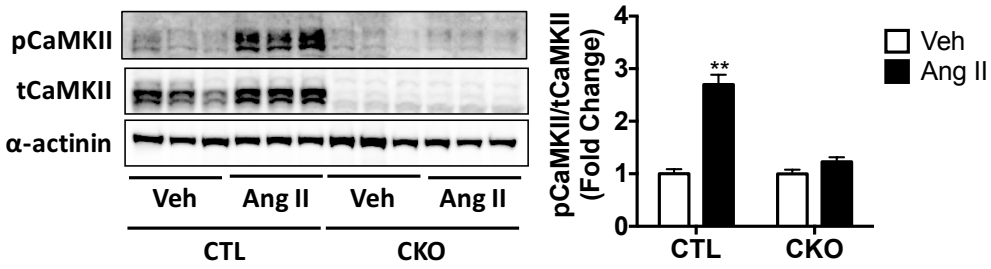

Figure A.5. Ang II activates CaMKIIδ in the isolated perfused heart. Western blot of phosphorylated CaMKIIδ in whole ventricular lysates of isolated control and CKO mouse hearts perfused with 1 μmol/L Ang II for 10 mins (n=3 each group). **P<0.01 vs Veh.

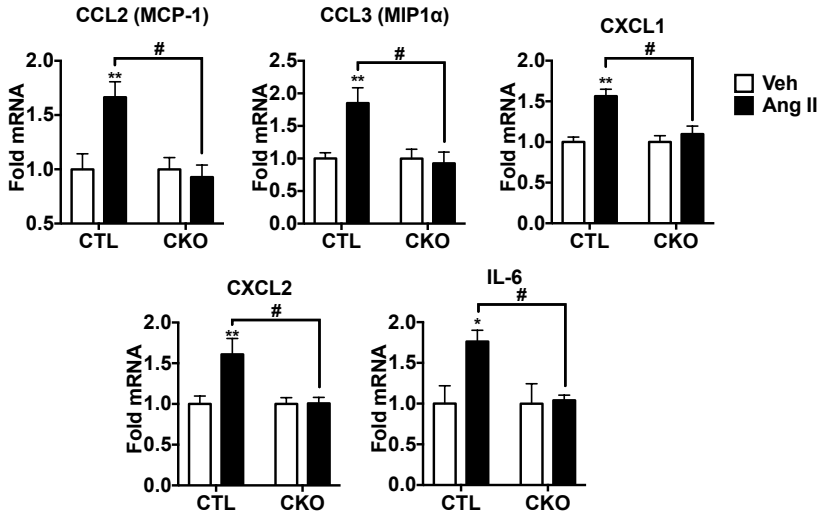

Figure A.6. Ang II induces CaMKIIδ-dependent inflammatory gene expression in isolated adult mouse ventricular cardiomyocytes *in vitro*. Inflammatory cytokine and chemokine mRNA as measured by qPCR in isolated AMVM treated with 1 μmol/L Ang II for 2 hours (n=4-6 each group). *P<0.05 vs Veh, **P<0.01 vs Veh, #P<0.05 CTL vs CKO.

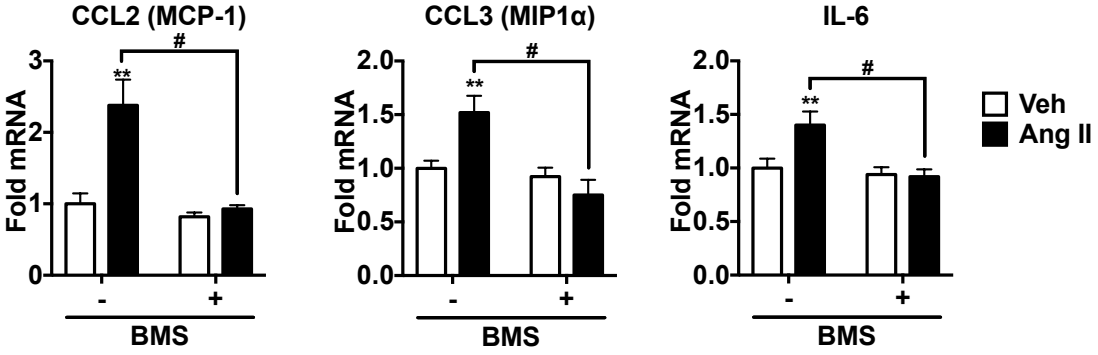

Figure A.7. Inflammatory gene expression induced by Ang II *in vitro* requires nuclear factor kappa B. Inflammatory cytokine and chemokine mRNA as measured by qPCR in isolated AMVM treated with 1 μmol/L Ang II for 2 hours ± 5 μmol/L BMS-345541 (n=4-6). **P<0.01 vs Veh, #P<0.05 Ang II + BMS vs Ang II alone.

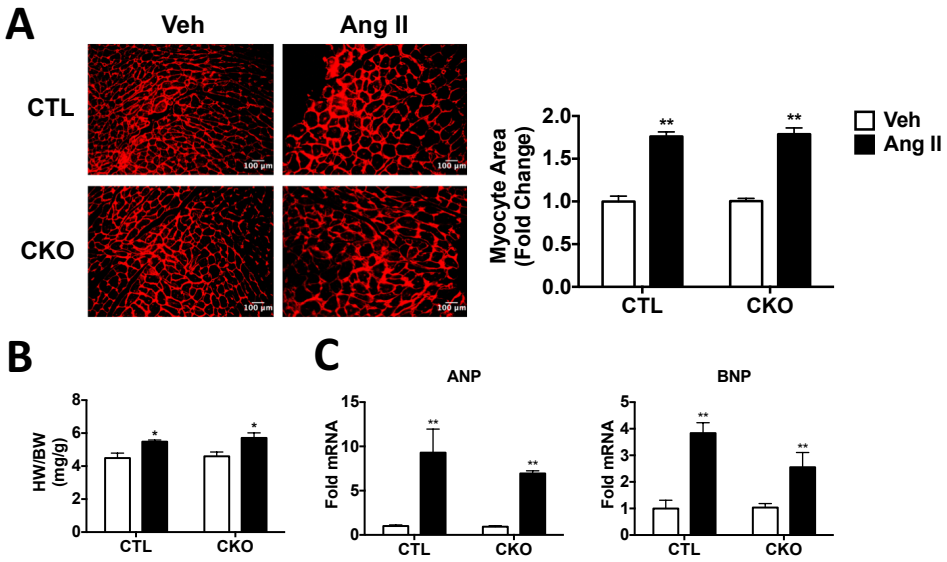

Figure A.8. Cardiac hypertrophy induced by 7 day Ang II infusion does not require CaMKIIδ. **A,** Representative pictures of cardiac sections stained with wheat germ agglutinin taken from mice infused with vehicle or Ang II for 7 days and quantitation of myocyte cross-sectional area. **B,** Heart weight-to-body weight ratio in control and CKO mice treated with vehicle or Ang II for 7 days. **C,** Hypertrophic gene markers atrial natriuretic peptide and brain natriuretic peptide as measured by qPCR in whole ventricular lysates taken from control and CKO mice infused with vehicle or Ang II for 7 days (n=3 each group). P*<0.05 vs Veh, P**<0.01 vs Veh.

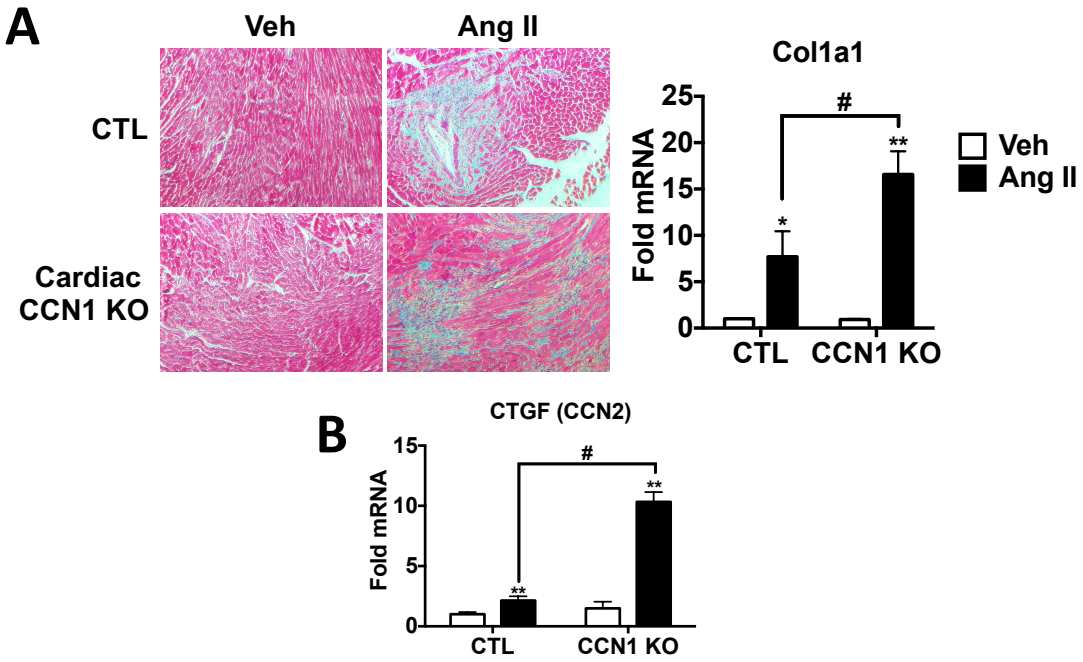

Figure A.9. Cardiomyocyte CCN1 deletion does not protect the heart from Ang II-induced fibrosis. **A,** Representative sections stained with Masson's Trichrome and Col1a1 mRNA as measured by qPCR in control and cardiomyocyte-specific CCN1 knockout mice infused with 1.5 μg/kg/min Ang II for 7 days. **B,** Connective tissue growth factor mRNA as measured by qPCR in whole ventricular lysates of mice infused with vehicle or Ang II for 7 days (n=3 each group). P*<0.05 vs Veh, P**<0.01 vs Veh, P#<0.05 CTL vs CCN1 KO.

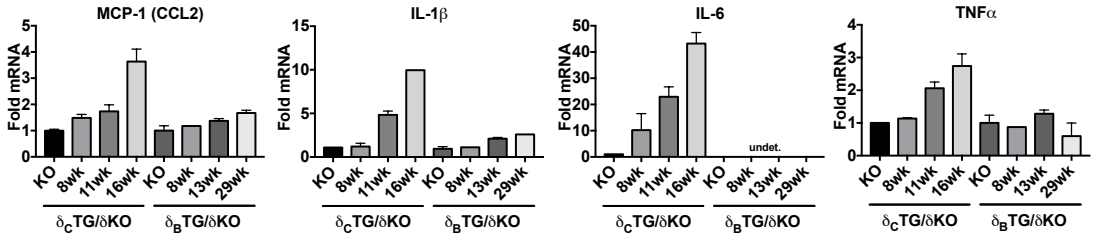

Figure A.10. Transgenic overexpression of the δ_C but not of the δ_B splice variant increases gene expression of multiple pro-inflammatory chemokines and cytokines (n=1-4 each).

Electronic cigarettes and cardiac inflammation

Our growing interest in cardiac inflammation led to a collaboration with Dr. Laura Crotty Alexander to investigate cardiac inflammation in an *in vivo* mouse model of electronic cigarette delivery. Electronic cigarettes are becoming increasingly popular and the effects of exposure to vapor produced by electronic cigarettes have been poorly investigated. In collaboration with the Alexander lab, we embarked on an *in vivo* mouse study to determine the effects of e-cigarette vapor (EV) on the heart. Preliminary data from our laboratory show that mice exposed to EV for 1 hour daily for 1 to 3 months do not have increased inflammatory gene expression and show only modest increases in fibrotic gene expression in the heart as compared to air controls (Fig. A.11 and A.12). Nicotine has been demonstrated to have direct anti-inflammatory effects on macrophages mediated through the alpha 7 nicotinic receptor[162]. Since the alpha 7 nicotinic receptor has also been demonstrated to desensitize in response to chronic stimulation with nicotine[163, 164], we hypothesized that chronic exposure to nicotine *in vivo* should potentiate inflammatory responses to pro-inflammatory stressors such as LPS. We observed, however, that hearts of mice infused subcutaneously with nicotine or saline for 2 weeks showed equivalent increases in inflammatory genes induced by intraperitoneal LPS injection

(Fig. A.13). Also, while nicotine treatment *in vitro* inhibited the inflammatory response of peritoneal macrophages isolated from saline-infused mice to LPS, this response was not altered in peritoneal macrophages isolated from nicotine-infused mice (Fig. A.14). We performed similar experiments using peritoneal macrophages isolated from mice exposed to EV or air and observed similar results as in the nicotine infusion experiments (data not shown).

Although negative, the preliminary results presented in this section of the dissertation do not rule out the possibility of an effect of chronic EV exposure on inflammatory responses in the heart. Mice were exposed to EV once daily for an hour, an experimental approach that may be too intermittent and acute to induce changes. We are currently designing studies to expose mice to EV up to 3 times daily.

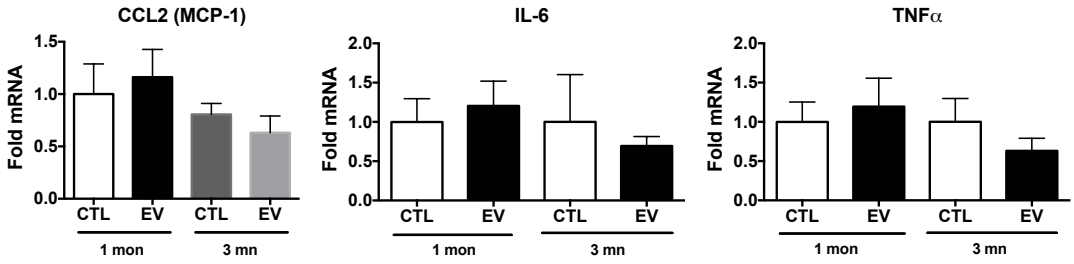

Figure A.11. Inflammatory cytokine and chemokine gene expression in the heart is not induced by electronic cigarette vapor exposure. Inflammatory cytokine and chemokine mRNA as measured by qPCR in hearts of wild type mice exposed to electronic cigarette vapor daily for 1 and 3 months (n=3-9 each group).

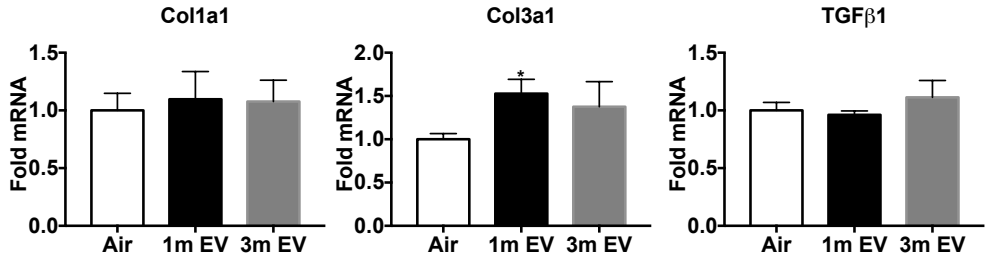

Figure A.12. Fibrotic gene expression is moderately increased in whole hearts of mice exposed to electronic cigarette vapor for 1-3 months. Fibrotic gene markers in whole ventricular lysates of mice exposed to electronic cigarette vapor daily for 1 and 3 months as measured by qPCR (n=3-9 each group). *P<0.05 vs Air.

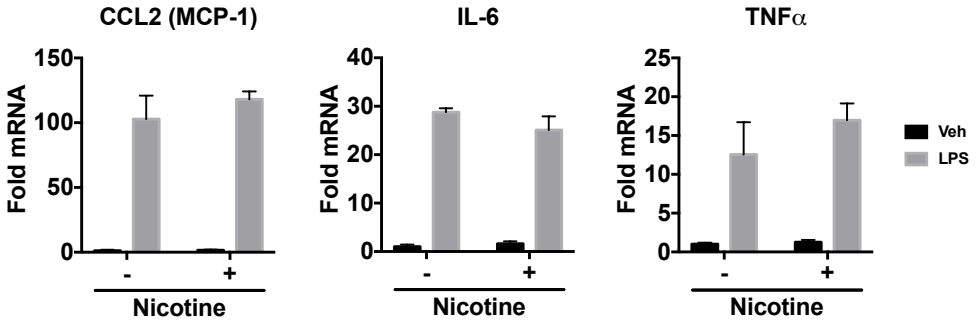

Figure A.13. Chronic nicotine infusion does not alter inflammatory gene expression induced in the heart by intraperitoneal LPS injection. Inflammatory cytokines and chemokines as measured by qPCR in ventricular lysates from mice infused with saline or 1.5 mg/kg/d nicotine for 2 weeks and treated with 10 mg/kg LPS i.p. 8 hours before sacrifice (n=2-4 each group).

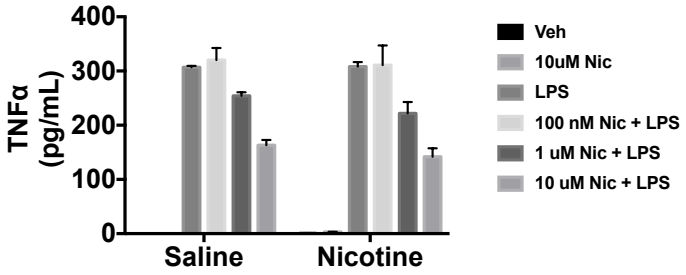

Figure A.14. TNFa release stimulated by *in* vitro LPS treatment in peritoneal macrophages is inhibited by nicotine but not altered in macrophages isolated from mice infused with nicotine. TNFa measured in media taken from peritoneal macrophages isolated from mice infused with saline or 1.5 mg/kg/d nicotine for 2 weeks and treated with 200 ng/mL LPS *in vitro* for 2 hours (n=2 each group).

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