CaMKIIδ Mediates Myocardial Ischemia/Reperfusion Injury Through NF-κB

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Abstract

Rationale—Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been implicated as a maladaptive mediator of cardiac ischemic injury. We hypothesized that the inflammatory response associated with in vivo ischemia/reperfusion (I/R) is initiated through CaMKII signaling.

Objective—To assess the contribution of CaMKIIδ to the development of inflammation, infarct and ventricular dysfunction following in vivo I/R and define early cardiomyocyte-autonomous events regulated by CaMKIIδ using cardiac-specific knockout (KO) mice.

Methods and Results—Wild-type (WT) and CaMKIIδ KO mice were subjected to in vivo I/R by occlusion of the left anterior descending (LAD) artery for 1-hr followed by reperfusion for various times. CaMKIIδ deletion protected the heart against I/R damage as evidenced by decreased infarct size, attenuated apoptosis and improved functional recovery. CaMKIIδ deletion also attenuated I/R induced inflammation and upregulation of NF-κB target genes. Further studies demonstrated that I/R rapidly increases CaMKII activity, leading to NF-κB activation within minutes of reperfusion. Experiments using cyclosporine A and cardiac-specific CaMKIIδ knockout mice indicate that NF-κB activation is initiated independent of necrosis and within cardiomyocytes. Expression of activated CaMKII in cardiomyocytes lead to I kappa B kinase (IKK) phosphorylation and concomitant increases in nuclear p65. Experiments using an IKK inhibitor support the conclusion that this is a proximal site of CaMKII-mediated NF-κB activation.

Conclusions—This is the first study demonstrating that CaMKIIδ mediates NF-κB activation in cardiomyocytes following in vivo I/R and suggests that CaMKIIδ serves to trigger, as well as to

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sustain subsequent changes in inflammatory gene expression that contribute to myocardial I/R damage.

Keywords

Ischemic heart disease; myocardial inflammation; reperfusion injury; nuclear factor kappa B; Ca^{2+}/calmodulin-dependent protein kinase II

INTRODUCTION

Cardiac reperfusion following an acute myocardial infarction (MI) contributes to myocyte damage, generating what is referred to as myocardial ischemia/reperfusion (I/R) injury. Cardiomyocyte cell death in response to I/R results from increases in Ca^{2+} and reactive oxygen species (ROS) and is mediated through opening of the mitochondrial permeability transition (PT) pore[1, 2]. Subsequent to necrotic cardiomyocyte cell death, an inflammatory cascade which perpetuates further damage to cardiac tissue is activated[3–5]. One of the central players in inflammatory signaling is the transcription factor nuclear factor kappa B (NF-κB). Numerous reports have shown that NF-κB is activated following myocardial I/R[6–9]. Activated NF-κB translocates to the nucleus following release from its inhibitory subunit IκBα leading to transcriptional regulation of the expression of interleukins and cytokines. The NF-κB-mediated inflammatory cascade can be perpetuated through the ability of NF-κB targets such as TNFα and interleukins to further activate NF-κB signaling, as well as by the infiltration of neutrophils and other mononuclear inflammatory cells at sites of injury. The observation that cardiac specific overexpression of a dominant-negative IκBα[10] or inhibition of IκBα degradation[11] block NF-κB activation, reduce infarct size and preserve heart function in response to ischemia/reperfusion indicate that the activation of NF-κB within the cardiomyocyte is critical to this series of events. Pharmacological inhibitors of NF-κB activation also provide protection against cardiac I/R injury, attenuate the release of TNFα and IL-6, inhibit inflammation and apoptosis, reduce infarct size and improve functional recovery[12–14]. Thus inflammation mediated through NF-κB plays a critical role in infarct development following I/R.

While a maladaptive role of NF-κB in I/R injury is well documented, the initiating signals and cellular sites at which this response is triggered have not been fully elucidated. Ca^{2+}/calmodulin-dependent kinase II (CaMKII), a multifunctional heteromeric serine/threonine protein kinase, is activated by both Ca^{2+} and oxidative stress[15]. CaMKII has been demonstrated to mediate *ex vivo* I/R injury and cardiac remodeling after myocardial infarction based on experiments using pharmacological inhibitors or transgenic mice overexpressing a CaMKII inhibitory peptide[16–19]. CaM kinase has also been demonstrated to play a role in inflammatory gene expression in macrophages[20–22]. Our laboratory generated conventional knockout (KO) mice[23] and more recently cardiac specific knockout mice (CKO) in which the predominant cardiac CaMKII isoform, CaMKII delta (δ)[24–27] is deleted. The studies presented here use these CaMKIIδ KO mouse lines to test the hypothesis that CaMKII is required for the initiation of I/R induced NF-κB activation in cardiomyocytes and plays a critical role in the subsequent inflammatory responses that contribute to myocardial I/R damage.

METHODS

Please see detailed Methods in the Online Supplement which provide expanded details of in vivo I/R and hemodynamic measurements, heart tissue preparation and nuclear fractionation, TUNEL, DNA laddering, CaMKII and NF-κB activity assays, immunofluorescence staining and affymetrix gene array analysis. Generation of global CaMKIIδ KO mice was described previously[23]. Cardiac specific CaMKIIδ KO mice were generated by crossing heterozygous floxed CaMKIIδ mice[23] with MLC 2v-Cre mice[28] as detailed in the Online Supplement. Myocardial I/R was induced by left anterior descending (LAD) coronary artery occlusion for 1 hr followed by reperfusion for various times up to 24 hrs. All 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 UCSD.

Statistical analysis

Data are presented as mean ± SEM as indicated and were analyzed by 2-tailed Student's t test between 2 groups or by ANOVA when 3 or more groups were compared. P values of less than 0.05 were considered statistically significant.

RESULTS

Infarct size and heart function

To test the role of CaMKIIδ in myocardial tolerance to I/R injury, CaMKIIδ KO and WT mice were subjected to left anterior descending coronary artery ligation for 1 hr followed by reperfusion for various times. Using a direct kinase activity assay, we demonstrate that CaMKII is activated (Ca^{2+} independent "autonomous" activity is increased) in WT mice at 3-min reperfusion following 1-hr in vivo ischemia (Figure 1A). As a further readout of CaMKII activation we measured phosphorylation of two substrates, the type two ryanodine receptor (RyR2) and phospholamban (PLN), at their CaMKII-specific phosphorylation sites. Marked time-dependent increases in RyR2 Ser2814 and PLN Thr17 phosphorylation were observed following reperfusion in WT but not in CaMKIIδ KO mice (Figure 1B).

Susceptibility to and recovery from I/R injury was then assessed. The area at risk (AAR) was not different in CaMKIIδ KO and WT littermates but infarct size following I/R was significantly reduced in the KO hearts (Figure 1C; infarct size/AAR: 46.5±7.5% in WT vs. 32.8 ± 4.3% in KO, p<0.05). The decreased infarct was accompanied by improved functional recovery. Maximal left ventricular pressure (LVP_{max}), dP/dt_{max} and dP/dt_{min} were not different in WT or KO mice at baseline[23] or under sham conditions (Online Table I and Figure 1 legend). Values for dP/dt_{max} and dP/dt_{min} measured at 24-hr reperfusion were, however, both significantly greater (p<0.05) in KO than in WT mice (Figure 1D). The difference in dP/dt_{max} was likely not due to altered preload since LVEDP was normal and did not differ between the two groups (Online Table I). The improved dP/dt_{min} observed following I/R in the KO mice may have reflected the higher LVP_{max} in this group since tau, a preferable measure of global LV relaxation, did not differ between WT and KO mice.

Apoptosis and inflammation

It is well accepted that cardiomyocyte cell death contributes to I/R injury. We examined the extent of apoptosis at 24-hr reperfusion following 1-hr ischemia. Apoptosis assessed by TUNEL staining (Figure 2A), DNA laddering (Figure 2B) and the level of cleaved caspase-3 (Figure 2C) were significantly greater in WT than in KO mice. Inflammation, a recognized consequence of myocardial ischemic insult, was evidenced by enhanced mononuclear cell infiltration as assessed by CD68 staining. The increase in CD68 staining observed in WT hearts at 24-hr reperfusion was significantly diminished by CaMKIIδ deletion (Figure 2D). The expression of monocyte chemotactic protein-1 (MCP-1/CCR2) was also significantly increased in hearts of WT but not in KO mice following I/R (Figure 2E).

NF-κB activation

The NF-κB family of transcription factors plays a central role in regulating the expression of inflammatory and cell death genes linked to cardiovascular pathology. We tested the hypothesis that NF-κB activation in response to I/R is regulated through CaMKIIδ. An early event in NF-κB activation is the degradation of IκBα. In WT mice, reperfusion following 1-hr ischemia was accompanied by a rapid and transient decrease in IκBα expression; this response was seen as early as 5 minutes after reperfusion and prevented by CaMKIIδ deletion (Figure 3A). Subcellular fractionation of hearts isolated at various times after I/R demonstrated increases in the p65 NF-κB subunit in nuclear fractions from WT but not KO mice (Figure 3B). Finally we assessed NF-κB "activity" in ventricular lysates through binding to its consensus sequence (TransAM NF-κB oligonucleotide). NF-κB binding was not changed during ischemia, but quickly increased in WT hearts at 5 minutes and at 2 hrs of reperfusion; this increase was sustained throughout 24 hrs of reperfusion. (Figure 3C). In contrast, activation of NF-κB was not significantly increased in KO hearts at any time following reperfusion (Figure 3C).

To explore the dependence of NF-κB activation on opening of the mitochondrial PT pore and development of necrosis, mice were treated with cyclosporin A (CsA 10mg/kg, administrated intravenously 10 min before reperfusion). CsA treatment reduced cytochrome C release into cytosolic fractions following I/R, indicating effective PT pore inhibition (Figure 4A). Nuclear p65 translocation in response to I/R was, however, unchanged by CsA treatment (Figure 4B). This observation suggests that I/R can induce NF-κB activation independent of necrotic damage to mitochondria, consistent with the very rapid onset of NF-κB activation.

Transcriptome analysis of I/R injury in WT and CaMKIIδ hearts

We used Affymetrix Gene arrays to compare changes in mRNA levels at 24-hrs reperfusion following 1-hr ischemia in WT and KO mouse hearts. There were 3129 differentially expressed transcripts in WT versus CaMKIIδ KO after I/R (interaction $P<0.05$ and minimum fold between groups > 20%) as shown in Figure 5A. The 5kb promoters of these differentially expressed genes were scanned for enrichment of evolutionarily conserved (human to mouse) transcription factor binding sites using the Whole Genome RVista tool[29]. Transcription factor binding sites enriched in the differentially regulated genes included

those for the known CaMKIIδ target MEF2c (–log10, P=12.3)[30] and those for NF-κB (–log10 P=10.3). There were 313 predicted NF-κB target genes upregulated in WT hearts which were significantly suppressed or downregulated in CaMKIIδ KO hearts (Online Table II). Many of these genes clustered by Gene Ontology (GO) analysis into categories related to apoptosis, cytokine signaling and regulation of NF-κB signaling. A diagrammatic representation including some of the I/R regulated, CaMKII dependent NF-κB target genes is shown in Figure 5B.

CaMKII signaling to NF-κB

To directly demonstrate and determine the mechanism for CaMKII effects on NF-κB activation we expressed constitutively active CaMKIIδ in neonatal rat cardiomyocytes. The amount of nuclear p65 was increased in cells expressing CaMKIIδ (Figure 6A). In addition we determined that I kappa B kinase (IKK), a proximal kinase in the NF-κB signaling cascade, was phosphorylated when CaMKII activity was increased (Figure 6B). Pharmacological inhibiton of IKK with BMS-345541 blocked the ability of CaMKII to increase nuclear p65 (Figure 6A). These data suggest that CaMKII activates IKK to initiate NF-κB signaling. To test the importance of IKK activation and NF-κB signaling using the in vivo I/R injury model, BMS-345541 was administrated to mice intravenously at 2mg/kg 10 min before reperfusion. The efficacy of IKK inhibition was evidenced by the ability of this drug to significantly decrease I/R induced NF-κB activation in WT mice (Figure 6C, third vs. fourth bars). Treatment of WT mice with BMS-345541 lead to a 31% decrease in infarct size (Figure 6D). In contrast the inhibitor did not further reduce I/R induced NF-κB activation or infarct size in KO mice (Figure 6C,D). The lack of additive protection by CaMKIIδ deletion plus NF-κB inhibition is consistent with there being a common mechanism underlying the benefit of deleting CaMKIIδ and of inhibiting NF-κB signaling. The finding that there is no additional benefit to CaMKII deletion when IKK is inhibited supports the cell based studies showing that CaMKII regulates NF-κB signaling in cardiomyocytes through IKK, rather than by regulating a more distal process in the NF-κB activation cascade.

Cardiac specific CaMKIIδ deletion and NF-κB signaling

The rapid activation of CaMKII and NF-κB suggests that this pathway is directly regulated by increases in Ca^{2+} and ROS signaling in cardiomyocytes. Nonetheless, it is possible that CaMKII regulates NF-κB signaling in inflammatory cells that are activated in or recruited to the heart during reperfusion. To directly demonstrate that the I/R protection afforded by CaMKIIδ deletion results from effects in the cardiomyocyte compartment we used cardiac specific CaMKIIδ KO (CKO) mice generated by crossing CaMKIIδ^{fl/fl} with MLC2v-Cre mice[28]. Consistent with our reported findings using global KO hearts[23], and indicative of selective loss of CaMKII activity in the CKO mice, the basal phosphorylation of RyR2 and PLN at their CaMKII, but not at their PKA, phosphorylation sites was significantly reduced (Online Figure IIA). Cardiac specific deletion of CaMKIIδ did not, however, alter ventricular chamber dimension, wall thickness or cardiac function as assessed by echocardiography in mice at 2 months of age (Online Figure IIB). Cardiac specific CaMKIIδ KO mice subject to in vivo I/R were protected to an extent similar to that observed in the global CaMKIIδ KO mice. Infarct size/AAR was reduced to 31.7±1.6% in CKO vs.

43.7±4.2% in Ctrl (fl/fl) mice (Figure 7A). The degradation of IκBα and activation of NF-κB observed at early times (5 to 30 min) after reperfusion was also significantly inhibited by cardiac specific CaMKIIδ deletion (Figure 7 B–C). In addition, the subsequent development of inflammation, assessed by CD68 staining at 24-hrs, was markedly attenuated in the cardiac specific CaMKIIδ KO mouse hearts (Figure 7D). Thus CaMKII signaling within the cardiomyocyte compartment initiates NF-κB activation and thereby contributes to I/R induced inflammation and infarct development.

DISCUSSION

The role of CaMKII in ischemic heart diseases

The current study provides evidence that CaMKIIδ is a mediator of the effects of I/R on IκBα degradation, NF-κB activation and upregulaton of putative NF-κB target genes and that its actions are mediated through IKK phosphorylation. This is the first report to utilize the cardiac specific CaMKIIδ KO mice generated in our laboratory. Studies using these mice provide evidence that deletion of CaMKIIδ within the cardiomyocyte results in no overt basal phenotype but markedly limits the ability of I/R to increase NF-κB activation. Concomitantly, CaMKIIδ gene deletion attenuates I/R induced cardiac inflammation, cardiomyocyte death, infarct development and loss of contractile function.

A series of studies from the Mattiazi group used the *ex vivo* isolated perfused heart to examine the involvement of CaMKII in global I/R injury[16, 17]. These studies concluded that the salutary effects of CaMKII inhibition result from preventing the disordered cardiomyocyte Ca^{2+} homeostasis that contributes to cell death and mechanical dysfunction following I/R. A primary target of CaMKII action was suggested by these studies to be the phosphorylation of phospholamban (PLN). We demonstrate that in vivo I/R increases phosphorylation of PLN and ryanodine receptor (RyR2) at CaMKII sites in WT but not in CaMKIIδ KO mice (Figure 1B). Thus I/R induced activation of CaMKII and phosphorylation of cytosolic Ca^{2+} handling proteins could contribute to mitochondrial dysfunction and subsequent necrotic cell death. Importantly, however, our data demonstrate that there is also a rapid CaMKII-mediated activation of NF-κB which is not secondary to necrosis and, while it may initially be protective[31], sets into motion a subsequent series of proinflammatory processes. Furthermore whereas the *ex vivo* model used in the studies cited above can only assess short term responses, in vivo I/R brings into play a more physiologically complex and prolonged sequence of events. Reperfusion following ischemia in vivo is associated with influx of leukocytes and activation of endogenous inflammatory cells that contribute to development of apoptosis and cardiac inflammation. The studies presented here indicate that averting the early and proximal cardiomyocyte autonomous activation of NF-κB by CaMKIIδ deletion also significantly attenuates these more chronic inflammatory responses as well as the ventricular dysfunction observed 24 hrs after reperfusion.

In studies exploring the role of CaMKII in myocardial infarction, Singh et al linked CaMKII to inflammation, specifically focusing on complement factor B (CFB) gene expression and the deleterious effects of LPS and TNFα[19]. CaMKII activation was shown to be required for CFB expression in response to LPS and TNFα in isolated cardiomyocytes. Further studies

demonstrated that LPS-mediated activation of toll-like receptors, which contributes to cardiac NF-κB activation[5, 32, 33], leads to CaMKII oxidation and activation in response to myocardial infarction[34]. The notion that increases in TLR and MyD88 activation lead to sustained CaMKII oxidation, contributing to NF-κB-mediated inflammatory gene expression following myocardial infarction, is wholly compatible with our data showing CaMKII-mediated increases in proinflammatory gene expression following in vivo I/R. What is most compelling, however, is that our studies also suggest that CaMKII is an initiator of inflammation through its direct role in NF-κB activation in the cardiomyocyte. Thus CaMKII serves as a trigger as well as an intermediate player in a sustained myocardial inflammatory cycle.

The role of CaMKII in NF-κB activation

As indicated in the introduction, NF-κB has been extensively implicated in myocardial I/R injury. There is abundant evidence that blocking NF-κB signaling limits infarct size, reduces neutrophil invasion and decreases inflammatory gene expression[10–14, 35]. Activation of NF-κB proceeds through a number of distinct pathways that converge on the phosphorylation and subsequent proteasomal degradation of the IκB inhibitor protein. Degradation of this protein facilitates the translocation of NF-κB dimers to the nucleus where they bind target genes and activate transcription. In our studies comparing WT and CaMKIIδ KO mice we report, consistent with published studies[12], that I/R leads to a rapid decrease in IκBα levels in WT mice. We demonstrate that IκBα degradation is attenuated by CaMKIIδ deletion suggesting that CaMKII either affects steps upstream of its phosphorylation or alters its proteasomal processing. We further show that both NF-κB nuclear translocation and it transcriptional activity are regulated through CaMKIIδ following I/R.

Our studies using BMS-345541, an inhibitor of IKK, the kinase responsible for IκB phosphorylation and its subsequent degradation, confirm the important role of NF-κB activation in infarct development following I/R. The data showing that pharmacological blockade of IκB kinase has no additional ameliorative effect when CaMKIIδ is deleted further imply that a common mechanism underlies the beneficial effects of blocking CaMKII and NF-κB signaling and suggests that NF-κB activation is directly downstream of CaMKII. Previous studies using neonatal rat cardiomyocytes and macrophages provided evidence that CaMKII can regulate NF-κB signaling[19, 36, 37]. Specifically, in neonatal rat cardiomyocytes CaMKIIδ overexpression was shown to increase IκBα degradation and NF-κB-dependent promoter/luciferase activity[36]. We show here that a more proximal event, the activation of IKK, is regulated through CaMKIIδ in these cells. Whether IKK is a direct target of CaMKII-mediated phosphorylation remains to be determined. Nonetheless the finding that inhibiting IKK blocks the ability of CaMKII to elicit NF-κB activation in NRVMs and prevents further effects of CaMKII deletion on infarct development in the in vivo I/R model support the conclusion that IKK is the proximal site of CaMKII action.

NF-κB targets in CaMKII mediated I/R injury

We identified a significant number of genes with NF-κB binding sites in their 5' upstream promoters that are upregulated by I/R in WT but not in CaMKIIδ KO mice. Which of these genes contribute to I/R-induced and CaMKII-mediated cardiomyocyte cell death, infarct

development and inflammation is not yet known. Anderson's group focused on complement factor B (CFB) as a gene that responds to lipopolysaccharides (LPS) in a CaMKII dependent manner both *in vitro* and in vivo[19]. This is, however, only one of many genes detected in their gene arrays as CaMKII dependent[19] and only one of the many genes identified in our arrays as being both CaMKII dependent and potentially NF-κB regulated. We specifically examined changes in the NF-κB regulated chemokine MCP-1 as a marker of inflammation, and demonstrated that the increases in MCP-1 protein expression seen 24 hrs after I/R are prevented by CaMKII deletion. Interestingly MCP-1 not only participates in chemoattraction but also regulates cell death pathways[38], thus MCP-1 is a potential mediator through which NF-κB signals contribute to cardiomyocyte death and infarct expansion. Undoubtedly a multitude of proinflammatory gene products conspire to induce cardiomyocyte injury and contractile dysfunction following I/R. The profound ameliorative effect of CaMKIIδ deletion on the expression of myriad NF-κB target genes categorized as regulators of cytokine signaling and apoptosis implicates CaMKII as a nodal regulator of this process.

In summary, we demonstrate for the first time that CaMKIIδ mediates rapid NF-κB activation and the development of apoptosis and inflammation in response to I/R in the in vivo mouse heart. Our studies using cardiac specific CaMKIIδ KO mice reveal that the maladaptive effects of CaMKII signaling elicited by I/R are early events that occur in the cardiomyocyte compartment. The activation of NF-κB signaling is independent of necrosis and results in regulation of multiple NF-κB target genes. We suggest that oxidative stress or Ca^{2+} release during reperfusion initiate rapid CaMKIIδ activation which leads to cardiomyocyte autonomous IKK phosphorylation and NF-κB activation. The ensuing changes in cardiac gene expression and recruitment of infiltrating and resident inflammatory cells fuel the subsequent inflammatory cascade that contributes to further cardiomyocyte injury. Inhibiting CaMKII during reperfusion could provide significant therapeutic advantage by targeting an early causal event in this cascade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Nonstandard Abbreviations

I/R	ischemia/reperfusion
CaMKII	Ca^{2+}/calmodulin-dependent kinase II

KO	knockout
CKO	cardiac specific knockout
NF-κB	nuclear factor kappa B
TNFα	tumor necrosis factor alpha
IL- 6	Interleukin-6
LAD	left anterior descending
IS	infarction size
AAR	area at risk
LV	left ventricle
CsA	cyclosporin A
IKK	I kappa B kinase
RyR2	type 2 ryanodine receptor
PLN	phospholamban
PT pore	mitochondrial permeability transition pore

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Calcium/calmodulin dependent kinase (CaMKII) is activated by calcium and reactive oxygen species (ROS).

- Reperfusion of the ischemic heart is associated with increased myocardial calcium and ROS generation, which lead to cardiac dysfunction.

- NF-κB, a master regulator of inflammation, contributes to tissue injury induced by cardiac ischemia/ reperfusion (I/R.)

What New Information Does This Article Contribute?

- CaMKII is activated within minutes of reperfusion and is required for NF-κB activation following in vivo I/R.

- CaMKII activates NF-κB directly by phosphorylating its upstream kinase - IKK.

- Activation of NF-κB by CaMKII is an early, cardiomyoctyte autonomous response that is critical for long- term inflammation, myocardial tissue injury and contractile dysfunction.

The mechanisms responsible for NF-κB activation in response to cardiac ischemia/ reperfusion are unknown. I/R associated increases in Ca2+ or ROS should activate CaMKII, but direct evidence is lacking. We found that CaMKII activity is rapidly increased in response to reperfusion and that CaMKII is required for subsequent activation of NF-κB. We demonstrate that these responses occur in a cardiomyocyte in a necrosis-independent manner and that CaMKII increases NF-κB activity through phosphorylation of its upstream kinase IKK. Deletion of CaMKII in cardiomyocytes prevents I/R mediated increases in NF-κB target gene expression, leads to a decrease in infarct size, and improved ventricular function at 24 h after I/R. These findings suggest that CaMKII-mediated NF-κB activation is an initiating signal that occurs in cardiomyocytes and is responsible for subsequent I/R injury. Blocking CaMKII could ameliorate excessive NF-κB mediated responses associated with various inflammatory diseases and in particular prove to be a viable therapeutic target for preventing the deleterious effects of myocardial reperfusion.
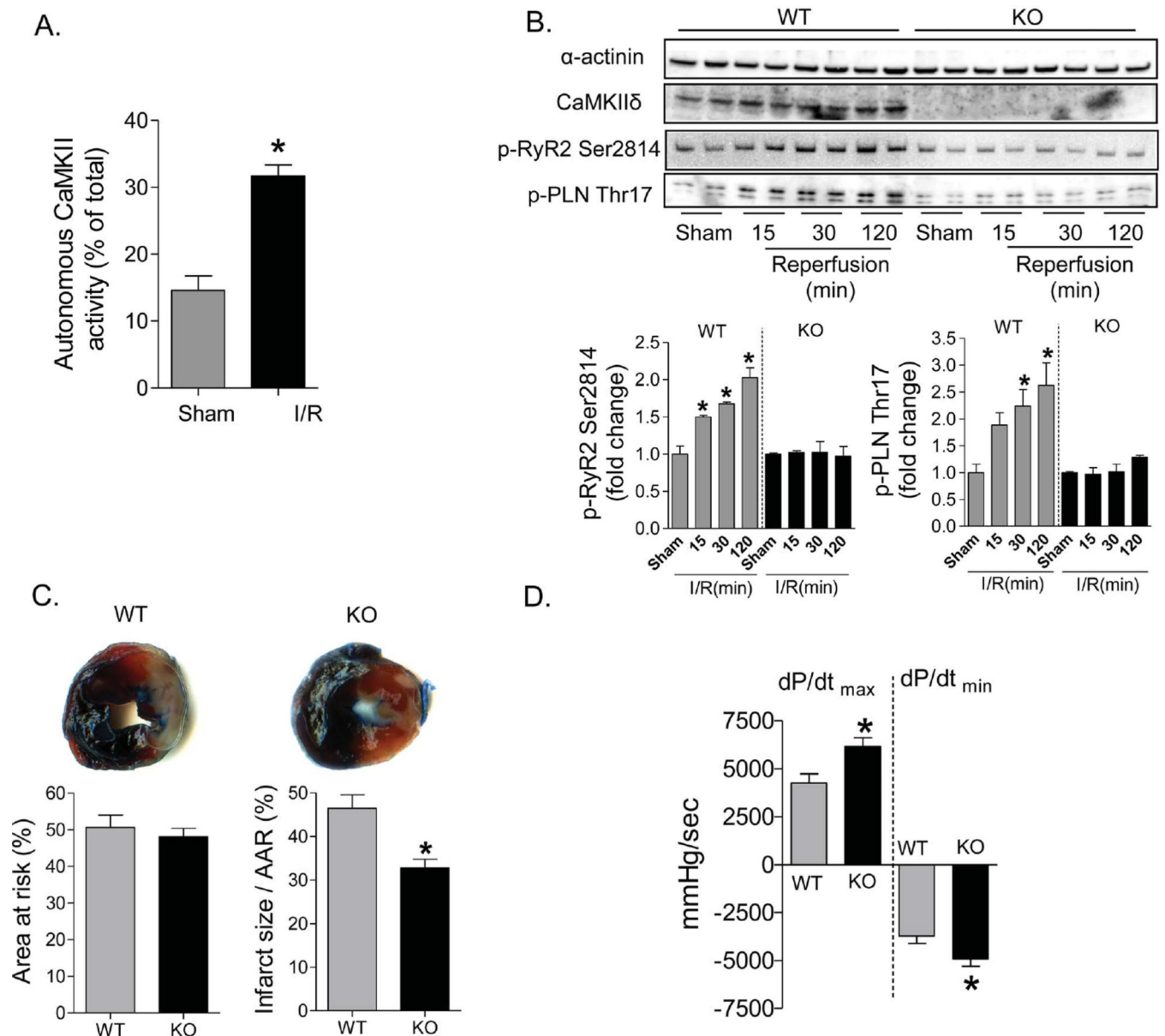
Figure 1. CaMKII is activated by in vivo myocardial ischemia/reperfusion and CaMKIIδ deletion reduces infarct size and enhances functional recovery

Animals were subjected to 1-hr ischemia followed by various times of reperfusion. (A) Increases in autonomous CaMKII activity were measured at 3-min reperfusion. Data are mean ± SEM of values from 3 mice. *p<0.05 versus Sham. (B) Phosphorylation of RyR2 and PLN at the CaMKII site was assessed in WT and CaMKIIδ KO mice at various times of reperfusion. Data are mean ± SEM of values from 3 determinations. *p<0.05 *vs* WT Sham. (C) Area at risk (AAR) and the ratio of infarct size to AAR were determined in WT and KO mice at 24-hr reperfusion. Data are mean ± SEM of values from 6 mice. (D) The maximum rates of rise and decline of left ventricular pressure (dP/dtmax and dP/dtmin) assessed at 24-hr reperfusion. Data are mean ± SEM of values from 8 mice. *p<0.05 versus WT. The dP/

dtmax and dP/dtmin values in sham-operated mice (~8500 and ~−6500 mmHg/sec respectively) were not significantly different for WT and KO.
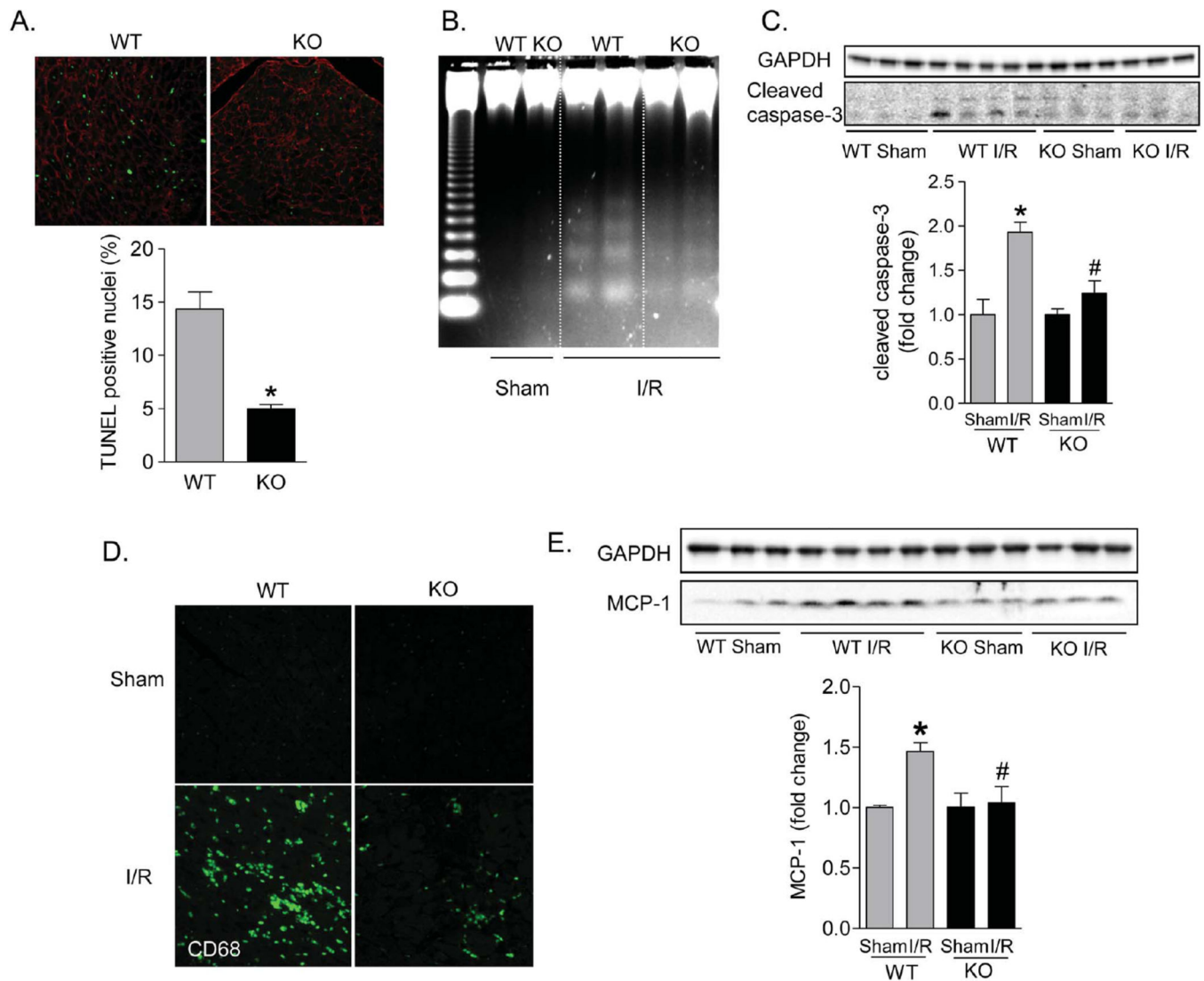
Figure 2. CaMKIIδ deletion reduces apoptosis and inflammation following in vivo ischemia/ reperfusion

Endpoints indicative of apoptosis and inflammation were assessed at 24-hr reperfusion following 1-hr ischemia. (A) TUNEL staining; apoptotic nuclei were stained (green) and cardiomyocytes were detected by wheat germ agglutinin staining (red). Original magnification is 20×. Data are mean ± SEM of values from 3 hearts per group, with at least 5,000 nuclei examined per heart. *$P < 0.05$ versus WT I/R. (B) DNA laddering. (C) Cleaved caspase-3 as detected by Western blotting. Data are mean ± SEM of values from 3–4 determinations. *p<0.05 *vs* Sham; #p<0.05 *vs* WT I/R. (D) Inflammatory cells were identified by CD68 staining. Original magnification is 20×. (E) MCP-1 expression levels measured by Western blotting. Data are mean ± SEM of values from 3–4 determinations. *p<0.05 *vs* Sham; #p<0.05 *vs* WT.

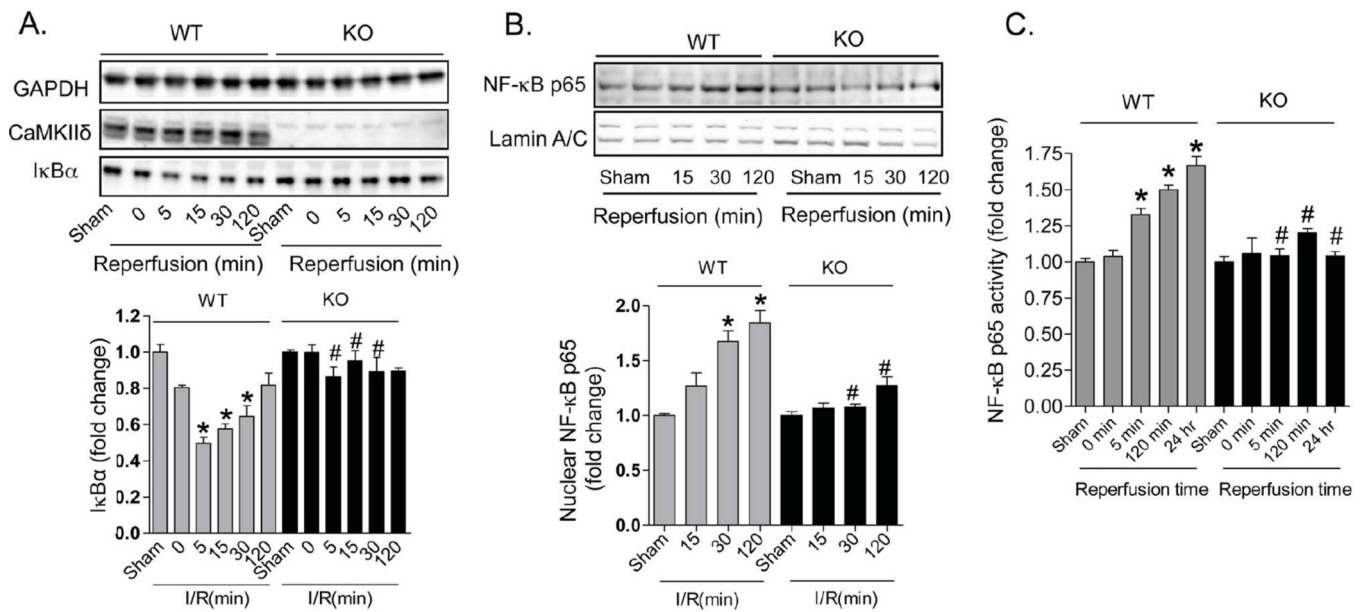**Figure 3. CaMKIIδ deletion inhibits IκBα degradation, NF-κB nuclear localization and NF-κB activity in response to in vivo I/R**

Animals were subjected to 1-hr ischemia followed by various times of reperfusion. (A) Degradation of IκBα was detected by Western blotting in hearts exposed to in vivo ischemia followed by various times of reperfusion. Data are mean ± SEM of values from 3–6 determinations. *p<0.05 vs Sham; #p<0.05 vs WT I/R. (B) Translocation of NF-κB p65 to the nucleus was detected by Western blotting following nuclear fractionation. Lamin A/C was used as a loading control. Data are mean ± SEM of values from 3–6 determinations. *p<0.05 vs Sham; #p<0.05 vs WT I/R. (C) NF-κB activity was measured by the TransAM oligonucleotide binding assay. Data are mean ± SEM of values from 3–6 samples. *p<0.05 vs Sham; #p<0.05 vs WT I/R.
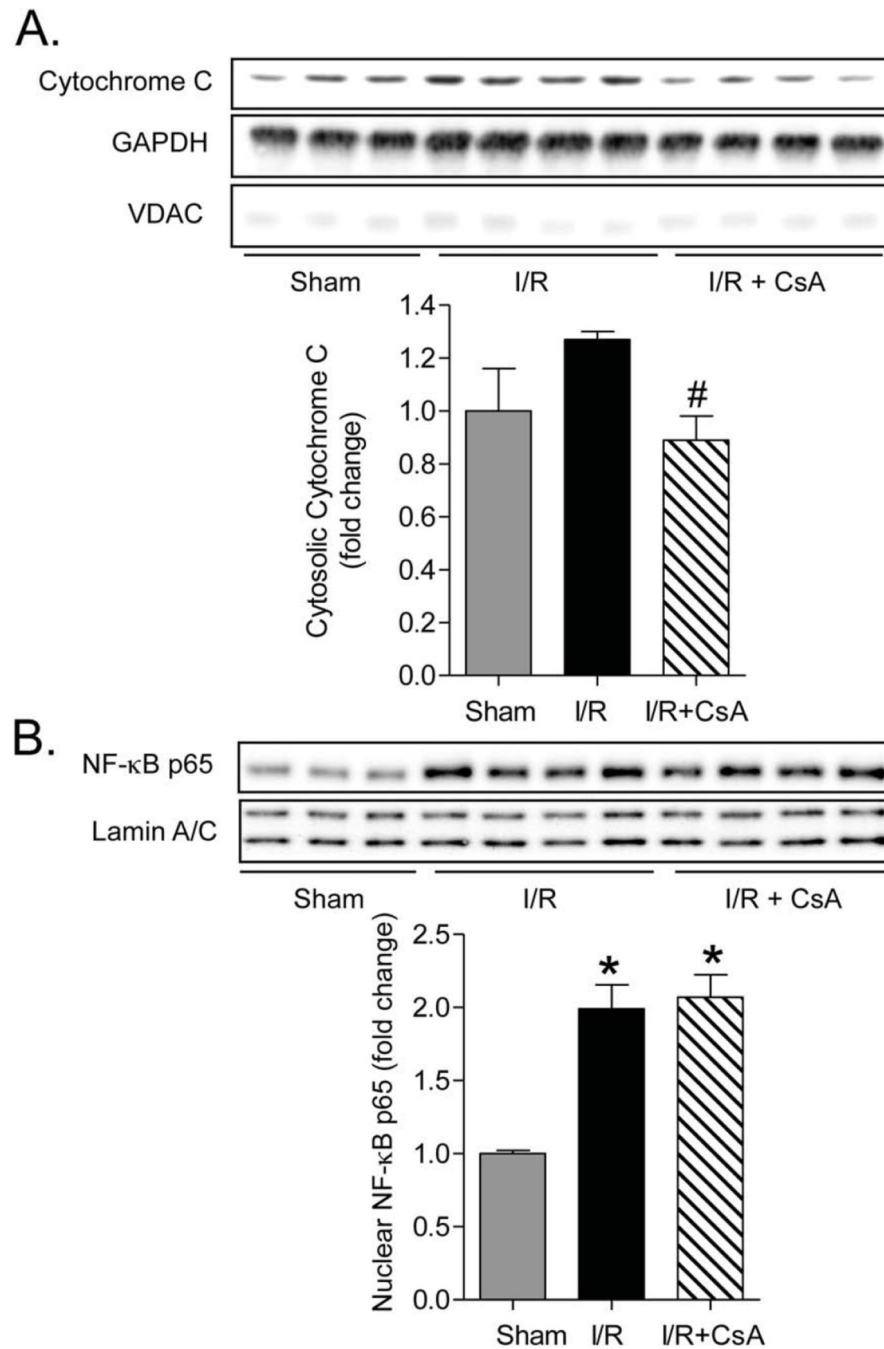
A.

B.

Figure 4. Inhibition of necrosis by CsA does not reduce NF-κB activation in response to in vivo I/R

Hearts were collected from mice treated ±CsA at 15-min reperfusion following 1-hr ischemia (A) Cytochrome C expression detected by Western blotting in mouse heart cytosolic fractions. GAPDH and VDAC were used as cytosolic and mitochondrial loading controls to assess purity of cytosolic fractions. Data are mean ± SEM of values from 3–4 determinations. #p<0.05 *vs* I/R (B) Translocation of NF-κB p65 to the nucleus was detected

by Western blotting in nuclear fractions from the same hearts. Lamin A/C was used as a loading control. Data are mean ± SEM of values from 3–4 mice. *p<0.05 versus Sham.
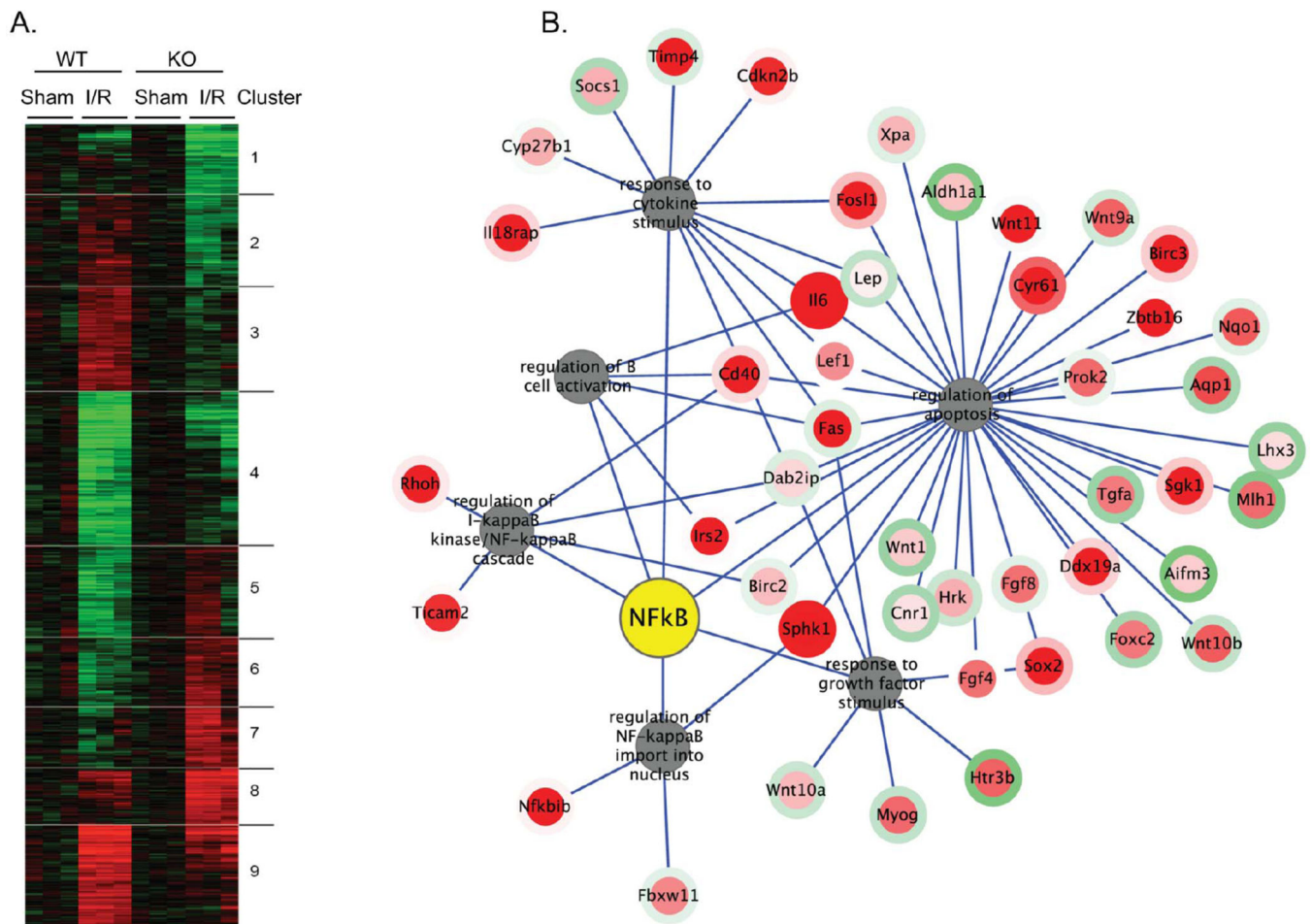
Figure 5. Clustering and functional annotation of transcripts differentially regulated by I/R in WT and CaMKIIδ hearts

(A) We identified 3129 transcripts that had a significant interaction (P<0.05) by genotype and I/R and clustered them with the HOPACH algorithm to identify major patterns of regulation. Rows represent probesets, columns are arrays; red indicates increase in expression and green indicates decreases in expression compared to baseline (sham) for each genotype (B) Network representation of selected gene ontology processes for the NF-κB target genes that are upregulated by I/R in WT versus CaMKIIδ KO hearts (i.e. putative NF-κB target genes from clusters 2, 3 and 9). Grey nodes represent gene ontology terms. Red and green nodes respectively represent genes upregulated and downregulated by I/R, with intensity correlated with the magnitude of the response. The central core color shows fold changes in WT hearts and the surrounding ring shows fold changes in CaMKIIδ KO hearts.
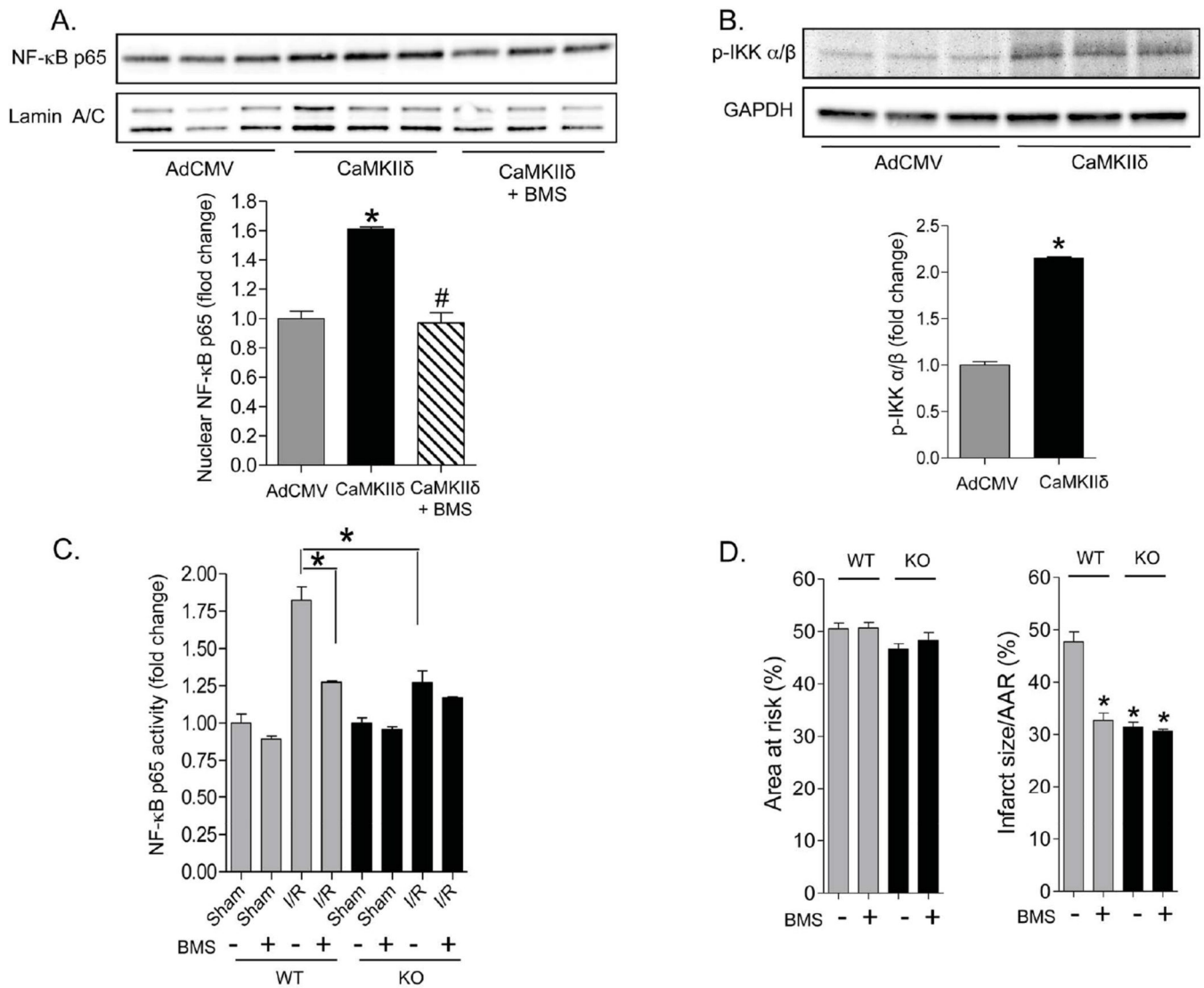
Figure 6. CaMKII induces I kappa B kinase (IKK) phosphorylation and inhibition of IKK reduces NF-κB activation and infarct development in response to in vivo I/R

For A and B, neonatal rat ventricular myocytes (NRVMs) were infected with adenovirus expressing GFP or constitutively active CaMKIIδ at 40 M.O.I. and harvested 24-hr after infection. (A) Translocation of NF-κB to the nucleus was detected by Western blotting following nuclear fractionation of cells treated ±15μM BMS-345541. Lamin A/C was used as a loading control. Data are mean ± SEM of values from 3 samples. *p<0.05 versus AdCMV; #p<0.05 *vs* CaMKIIδ. (B) Phosphorylated IKK was detected by Western blot. Data are mean ± SEM of values from 3 samples. *p<0.05 versus AdCMV. For C and D, hearts were collected at 24-hr reperfusion following 1-hr ischemia. (C) NF-κB activation by in vivo I/R measured by the TransAM oligonucleotide binding assay in WT and KO mice pretreated for 10 min with 2mg/kg BMS-345541 or vehicle. Data are mean ± SEM of values from 3 mice. *p<0.05 vs WT without BMS treatment. (D) Area at risk (AAR) and Infarct size to AAR ratio (IS/AAR) following in vivo I/R ±BMS treatment. Data are mean ± SEM of values from 4–6 determinations. *p<0.05 vs WT without BMS treatment.
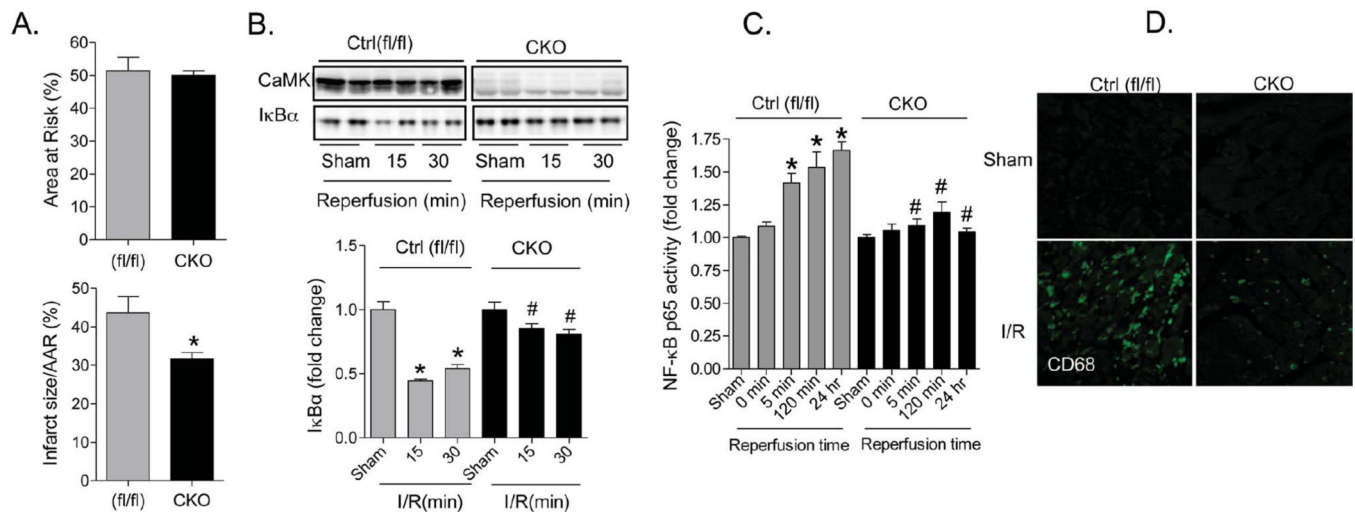
Figure 7. Cardiac specific deletion of CaMKIIδ protects against in vivo I/R injury
Animals were subjected to 1-hr ischemia followed by 24-hr reperfusion. (A) Area at risk (AAR) and Infarct size to AAR ratio (IS/AAR) in control and CKO hearts following I/R. Data are mean ± SEM of values from 4–5 determinations. *p<0.05 *vs* Ctrl I/R. (B) Degradation of IκBα detected by Western Blotting in control and CKO mouse hearts following I/R. Data are mean ± SEM of values from 3 determinations. *p<0.05 *vs* control Sham; #p<0.05 *vs* CKO Sham. (C) NF-κB activity was determined at the end of 1hr ischemia and after various times of reperfusion in control and KO hearts by TransAM oligonucleotide binding assay. Data are mean ± SEM of values from 3–4 determinations. *p<0.05 *vs* Sham; #p<0.05 *vs* Ctrl I/R. (D) CaMKIIδ deletion reduces inflammation following I/R. Inflammatory cells were identified by CD68 staining. Original magnification is 20×.