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

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ABSTRACT

Deletion of $Ca^{2+}$/calmodulin-dependent protein kinase II delta (CaMKIIδ) has been shown to protect against *in vivo* ischemia/reperfusion (I/R) injury. It remains unclear which CaMKIIδ isoforms and downstream mechanisms are responsible for the salutary effects of CaMKIIδ gene deletion. In this study we sought to compare the roles of the $CaMKII\delta_B$ and $CaMKII\delta_C$ subtypes and the mechanisms by which they contribute to *ex vivo* I/R damage. WT, CaMKIIδKO, and mice expressing only $CaMKII\delta_B$ or $\delta_C$ were subjected to *ex vivo* global ischemia for 25 min followed by reperfusion. Infarct formation was assessed at 60 min reperfusion by triphenyl tetrazolium chloride (TTC) staining. Deletion of CaMKIIδ conferred significant protection from *ex vivo* I/R. Re-expression of $CaMKII\delta_C$ in the CaMKIIδKO background reversed this effect and exacerbated myocardial damage and dysfunction following I/R, while re-expression of $CaMKII\delta_B$ was protective. Selective activation of $CaMKII\delta_C$ in response to I/R was evident in a subcellular fraction enriched for cytosolic/membrane proteins. Further studies demonstrated differential regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling and tumor necrosis factor alpha (TNF-α) expression by $CaMKII\delta_B$ and $CaMKII\delta_C$. Selective activation of $CaMKII\delta_C$ was also observed and associated with NF-κB activation in neonatal rat ventricular myocytes (NRVMs) subjected to oxidative stress. Pharmacological inhibition of NF-κB or TNF-α significantly ameliorated infarct formation in WT mice and those that re-express $CaMKII\delta_C$, demonstrating distinct roles for CaMKIIδ subtypes in I/R and implicating acute activation of $CaMKII\delta_C$ and NF-κB in the pathogenesis of reperfusion injury.



## 1. Introduction

The calcium/calmodulin dependent protein kinase II (CaMKII) is a dodecameric enzyme consisting of subunits encoded by four different genes known as CaMKIIα, β, γ, and δ. The predominant cardiac isoform is CaMKIIδ and it is alternatively spliced in the heart to generate $CaMKII\delta_B$ and $CaMKII\delta_C$ as well as other minor subtypes [1–5]. We have generated global and cardiac specific knockouts of CaMKIIδ and demonstrated that deletion of this protein ameliorates heart failure development in response to pressure overload, $G\alpha_q$ expression, and isoproterenol infusion [6–8]. We further reported that deletion of CaMKIIδ diminishes infarct development in response to *in vivo* ischemia/reperfusion [9]. Other studies using CaMKII inhibitory peptides or knock-ins of activation-deficient CaMKII have similarly concluded that CaMKII activation by a range of cardiac insults, including myocardial infarction, is deleterious [10–13]. In all of the aforementioned studies both the $CaMKII\delta_B$ and $CaMKII\delta_C$ subtypes of CaMKIIδ were genetically deleted or inhibited. Accordingly it is not yet known which subtype is responsible for the protective effect of ablating CaMKIIδ activity in the heart.

The studies reported here use mice in which CaMKIIδ deletion has been restored by crossing CaMKIIδKO mice with transgenic lines expressing either the $CaMKII\delta_B$ ($\delta_B$TG/δKO) or $CaMKII\delta_C$ ($\delta_C$TG/δKO) subtype [14]. This approach allowed us to examine the unique roles of

*Abbreviations:* Animals expressing only $CaMKII\delta_B$, ($\delta_B$TG/δKO); Animals expressing only $CaMKII\delta_C$, ($\delta_C$TG/δKO); BMS-345541, (BMS); $Ca^{2+}$/calmodulin-dependent protein kinase II delta, (CaMKIIδ); Cyclosporine-A, (CsA); Fractional shortening, (FS); Inhibitor of kappa B kinase, (IKK); Interleukin 6, (IL-6); Ischemia/reperfusion, (I/R); Left ventricular developed pressure, (LVDP); Neonatal rat ventricular myocytes, (NRVMs); Nuclear factor kappa-light-chain-enhancer of activated B cells, (NF-κB); Reactive oxygen species, (ROS); Receptor-interacting protein kinase 3, (RIP3); Sarcoplasmic reticulum, (SR); Triphenyl tetrazolium chloride, (TTC); Tumor necrosis factor alpha, (TNF-α).

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CaMKII$\delta_B$ and CaMKII$\delta_C$ in cardiomyocyte survival and infarct formation in response to I/R. We demonstrate that CaMKII$\delta_C$ expression reverses and exacerbates the diminished I/R damage observed in CaMKIIδKO mouse hearts whereas CaMKII$\delta_B$ expression further attenuates I/R damage. The difference in infarct development observed in $\delta_B$TG/δKO and $\delta_C$TG/δKO mice is associated with greater I/R-induced inhibitor of kappa B kinase (IKK) and NF-κB activation in $\delta_C$TG/δKO mice. CaMKII$\delta_C$-mediated NF-κB activation is recapitulated in NRVMs exposed to oxidative stress, and selective activation of CaMKII$\delta_C$ in a cytosol/membrane fraction is observed in NRVMs exposed to oxidative stress and in hearts exposed to I/R. TNF-α expression is also selectively increased in hearts from $\delta_C$TG/δKO mice following I/R. Blocking either IKK activation or TNF-α signaling diminished infarct development in $\delta_C$TG/δKO as well as in WT mice. These data suggest that selective activation of the CaMKII$\delta_C$ subtype in cardiomyocytes regulates cardiac-autonomous pro-inflammatory signaling events that contribute to ischemia/reperfusion injury.

## 2. Methods

### 2.1. Transgenic animals

Transgenic Black Swiss mice in which the predominant cardiac subtypes of CaMK, CaMKII$\delta_B$ and CaMKII$\delta_C$, are over expressed were generated in our laboratory and characterized as described [15,16]. Conventional CaMKIIδKO mice were generated and characterized as previously described [6]. CaMKII$\delta_B$ and CaMKII$\delta_C$ transgenic mice were crossed with conventional CaMKIIδKO mice to generate mice that express only CaMKII$\delta_B$ or CaMKII$\delta_C$. We refer to these animals as CaMKII$\delta_B$TG/δKO and CaMKII$\delta_C$TG/δKO. All mice used in the present study were male at 8 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.

### 2.2. 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 nM [$\gamma$-$^{32}$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.

### 2.3. Heart tissue fractionation procedure

Mice were killed by cervical dislocation, and hearts were quickly removed and placed in ice-cold PBS. Following cannulation, hearts were perfused with PBS to remove blood, frozen in liquid $N_2$, and pulverized. Tissue was then homogenized in isolation buffer (70 mmol/L sucrose, 190 mmol/L mannitol, 20 mmol/L HEPES, 0.2 mmol/L EDTA) that was supplemented with various inhibitors: sodium vanadate, leupeptin, aprotinin, *p*-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride. Homogenate was centrifuged at 600*g* for 10 m at 4 °C, and the supernatant transferred to another tube. The supernatant was centrifuged at 5000*g* for 15 m at 4 °C to yield a cytosol/membrane fraction. The pellet from the initial 600 g centrifugation was resuspended in nuclear lysis buffer (20 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 20 mmol/L HEPES, 200 nmol EDTA, 25% glycerol) and centrifuged at 600*g* for 10 m at 4 °C to yield a nuclear fraction.

### 2.4. Neonatal rat ventricular myocyte (NRVM) isolation and adenoviral infection

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1 to 2-day-old Sprague-Dawley rat pups, digested with collagenase, plated at density of $3.5 \times 10^4/cm^2$ and maintained overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), Prior to adenoviral Infection, isolated NRVMs were transferred to serum-free medium and infected with CaMKII $\delta_B$ or $\delta_C$ adenovirus at 10, 30, 50, 100, 300 multiplicity of infection (MOI) for 3 h. Cells were washed and maintained in serum-free medium for an additional 21 h prior to treatment with $H_2O_2$ or vehicle. To assess changes in nuclear and cytosolic/membrane fractions, NRVMs were fractionated according to a previously reported protocol [17]. Purity was determined using the cytosolic marker Rho GDP dissociation inhibitor (RhoGDI) and the nuclear marker Lamin A/C.

### 2.5. Transthoracic echocardiography

Echocardiography was performed using the VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described [18]. Body temperatures were maintained between narrow ranges (37.0 ± 1.0 °C) to avoid confounding effects of hypothermia.

### 2.6. Isolated perfused (ex vivo) I/R

Mice were killed by cervical dislocation, and hearts were quickly removed and placed in ice-cold $Ca^{2+}$-free Krebs-Henseleit buffer. Aortas were cannulated and hearts perfused by gravity flow on a Langendorff perfusion system (Radnoti LLC) at 37 °C and a constant pressure of 80 mm Hg with a modified Krebs-Henseleit buffer solution containing (in mmol/L); 2.0 $CaCl_2$, 130 NaCl, 5.4 KCl, 11 dextrose, 2 pyruvate, 0.5 $MgCl_2$, 0.5 $NaH_2PO_4$, and 25 $NaHCO_3$ and aerated with 95% oxygen and 5% carbon dioxide, pH 7.4. To measure infarct size, hearts were subjected to 25-minute global ischemia and 1-hour reperfusion; the ventricles were then frozen and cut transversely into 5 slices of equal thickness. The slices were then incubated in 1% TTC/PBS and fixed in 10% formalin-PBS for 24 h. Fixed slices were then scanned, and ImageJ was used to measure and calculate the size of the infarct area and the total area. For experiments utilizing BMS-345541 (Sigma-Aldrich) the drug was dissolved in Krebs-Henseleit buffer solution at a concentration of 5 μmol/L and was present throughout the I/R protocol. For those using etanercept, the drug was present for the entire I/R procedure at a concentration of 5 μg/mL. To assess cardiac function, a water-filled balloon connected to a pressure transducer (Gould Stathem *P*23 ID) was inserted into the left ventricle through the left atrium to monitor left ventricular developed pressure (LVDP); data collected using Powerlab, were processed with AD Instruments Chart 4 software (v4.12). Hearts were submersed in warm KHB (37 °C) throughout the perfusion. Functional recovery was expressed as a percentage of pre-ischemic LVDP.

### 2.7. Immunoblotting

Ventricular tissue was homogenized in RIPA buffer (10 mmol/L Tris-Cl (pH 8.0), 1 mmol/L EDTA, 0.5 EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mmol/L NaCl) that was supplemented with various inhibitors: sodium vanadate, leupeptin, aprotinin, *p*-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride. Western blot analysis was performed according to protocols described previously [9]. The antibodies for immunoblotting were as follows: CaMKIIδ (D.M. Bers, UC Davis); GAPDH (CST); phospho-IKKα/β at its autophosphorylation site (Ser176/180) (CST); phospho-CaMKII at its autophosphorylation site (Thr286) (Thermo); NF-κB p65 (CST); α-actinin (CST); RhoGDI (CST); Lamin A/C (CST); IκBα (CST)

### 2.8. RT-PCR

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

## 3. Statistical analysis

Data are presented as mean ± SEM as indicated and were analyzed by 2-tailed Student *t*-test between 2 groups or by ANOVA when 3 or more groups were compared. *P* values <0.05 were considered statistically significant.

## 4. Results

To examine the independent roles of the CaMKIIδ$_B$ and δ$_C$ subtypes in the heart we restored either δ$_B$ or δ$_C$ expression in a CaMKIIδ-null (KO) background. The resulting animals express only δ$_B$ (δ$_B$TG/δKO) or only δ$_C$ (δ$_C$TG/δKO). Survival of WT, KO, δ$_B$TG/δKO, and δ$_C$TG/δKO mice was assessed. Deletion of CaMKIIδ produced no overt phenotypic changes and did not affect survival relative to WT mice as shown previously [6]. δ$_B$TG/δKO animals also survived normally for at least 6 months. In contrast, the δ$_C$TG/δKO animals, like the previously studied δ$_C$TG [16], exhibited premature death with <20% survival by 21 weeks (Fig. 1A). Expression of the CaMKII δ$_B$ and δ$_C$ subtypes also had markedly different effects on *in vivo* cardiac function. Echocardiography on 6–8 week old mice revealed that fractional shortening (FS) was decreased by 63% in δ$_C$TG/δKO mice compared to WT mice (Fig. 1B) while δ$_B$TG/δKO animals did not display cardiac dysfunction.

We previously demonstrated that CaMKIIδ deletion attenuates I/R injury in response to *in vivo* left anterior descending coronary artery occlusion and subsequent reperfusion [9]. To examine the cardiac-intrinsic role of CaMKIIδ in I/R we performed *ex vivo* I/R experiments on isolated perfused hearts from 8-week-old mice. Infarct formation following 25 min ischemia and 1 h reperfusion was determined by TTC staining of heart sections. In WT animals, *ex vivo* I/R induced infarcts comprising 36.2 ± 2.5% of the cross-sectional area. Infarcts were significantly smaller, only 24.1 ± 1.4%, in CaMKIIδKO mouse hearts (Fig. 2A, B). Thus regulation of infarct development by CaMKII is evident not only *in vivo* but also in an *ex vivo* I/R model.

Hearts from δ$_B$TG/δKO mice were then examined and found to be protected against *ex vivo* I/R damage, with infarcts measuring 12.2 ± 1.9% of cross-sectional area. Conversely, in δ$_C$TG/δKO mouse hearts, the protective effect of CaMKIIδ gene deletion was lost with infarcts measuring 45.2 ± 1.8% of the cross-sectional area, significantly larger than those of WT, KO, and δ$_B$TG/δKO (Fig. 2A, B). Assessment of left ventricular developed pressure (LVDP) recovery during reperfusion confirmed that expression of CaMKIIδ$_C$ in CaMKIIδKO mice exacerbates I/R damage while expression of CaMKIIδ$_B$ does not (Supplemental Fig. 1).

CaMKII is known to be activated during *ex vivo* I/R [19,20], but previous experiments have not assessed subtype-specific CaMKII activation. Although we previously demonstrated that δ$_B$ and δ$_C$ can be equivalently activated by several pharmacological interventions [14], we wondered if I/R might lead to differential activation of the two subtypes. To assess I/R-induced activation of CaMKII we analyzed phosphorylation of CaMKII at its autophosphorylation site threonine-286. The increases in CaMKII autophosphorylation observed in whole cell lysates from hearts of δ$_B$TG/δKO and δ$_C$TG/δKO mice subjected to I/R were equivalent (Fig. 3A).

Many substrates of CaMKIIδ$_C$ (e.g. phospholamban, RyR2, and histone deacetylase 4) localize to the cytosol or the cytosolic face of the cardiac sarcoplasmic reticular (SR) membrane. Thus we carried out further studies using a subcellular fraction enriched for cytosolic and membrane proteins. Strikingly in this fraction we observed differential activation of the δ$_B$ and δ$_C$ subtypes in response to I/R. Indeed whereas no increases in CaMKII autophosphorylation were observed in cytosolic/membrane fractions from δ$_B$TG/δKO mouse hearts, there was a >3-fold increase in autophosphorylated CaMKII in cytosolic/membrane-enriched fractions from δ$_C$TG/δKO hearts (Fig. 3B).

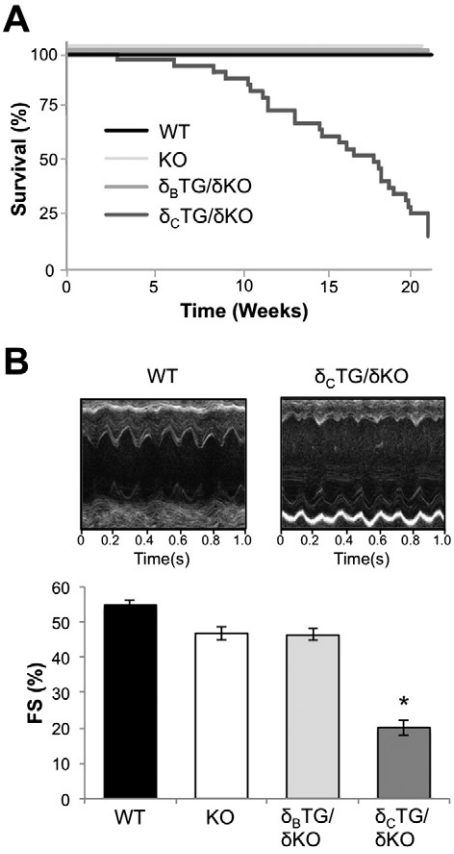


**Fig. 1.** CaMKIIδ$_C$TG/δKO but not CaMKIIδ$_B$TG/δKO mice display diminished survival and cardiac function. A, Kaplan-Meier analysis of survival of WT, CaMKIIδKO, δ$_B$TG/δKO, and δ$_C$TG/δKO mice, B. Representative echocardiographic recordings from WT and δ$_C$TG/δKO mice and quantification of fractional shortening (FS) measured in 6–8-week-old WT, KO, δ$_B$BTG/δKO, and δ$_C$TG/δKO mice. Data are mean ± SEM values from 4 to 6 mice. *$P < 0.05$ *vs* WT.

Our earlier studies examining *in vivo* I/R damage linked the deleterious effects of CaMKII to activation of IKK and subsequent NF-κB nuclear accumulation [9]. To determine which CaMKIIδ subtype was responsible, and also to determine if CaMKIIδ-mediated NF-κB activation can occur in the absence of systemic factors (*e.g.* leukocyte infiltration), we examined regulation of IKK and NF-κB by I/R in the *ex vivo* heart. The phosphorylation of IKK was found to be elevated during *ex vivo* reperfusion in δ$_C$TG/δKO mice but not in δ$_B$TG/δKO mice (Fig. 4A). Furthermore I/R-mediated activation of IKK in δ$_C$TG/δKO mice was associated with an increased nuclear localization of the p65 subunit of NF-κB, which was not observed in δ$_B$TG/δKO animals (Fig. 4B).

Nuclear p65 translocation would be expected to result in transcriptional activation of NF-κB target genes. Thus as further evidence that differences in NF-κB activation in δ$_C$TG/δKO and δ$_B$TG/δKO are functionally significant, we measured mRNA levels of genes regulated by NF-κB. Ischemia/reperfusion increased interleukin 6 (IL-6) mRNA in hearts from δ$_C$TG/δKO mice to a greater extent than in those from δ$_B$TG/δKO mice (Fig. 5A). Even more striking was the robust increase in TNF-α expression in δ$_C$TG/δKO mice and the absence of upregulation of this gene in δ$_B$TG/δKO animals (Fig. 5B).

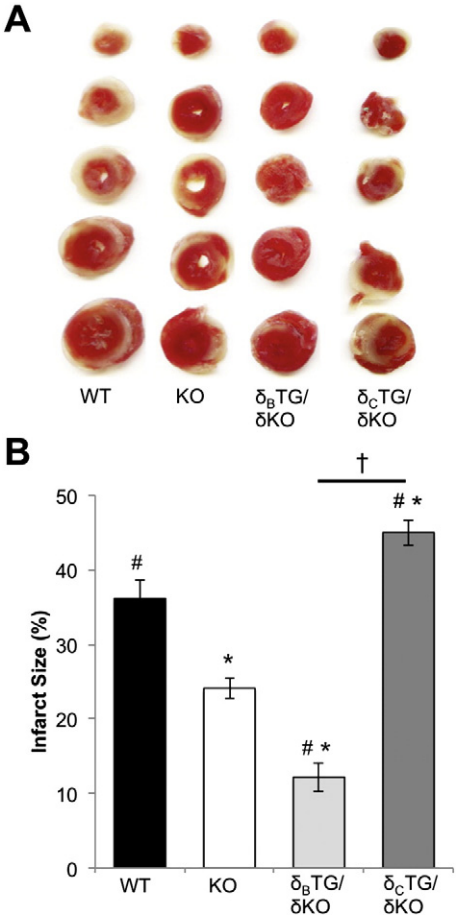


**Fig. 2.** Differential effects of CaMKII subtypes on I/R injury and oxidative stress. A, Representative images hearts from WT, CaMKIIδKO. CaMKII$\delta_B$TG/δKO. and CaMKII$\delta_C$TG/δKO mice subjected to 25 min ischemia and 1 h of reperfusion in the Langendroff mode. Hearts were sectioned and stained with TTC to reveal infarcted tissue. B, Infarct size was quantified from TTC stained heart sections. Data are mean ± SEM values from 14 to 16 mice. *$P < 0.05$ *vs* WT. #$P < 0.05$ *vs* KO. †$P < 0.05$ *vs* $\delta_C$TG/δKO.

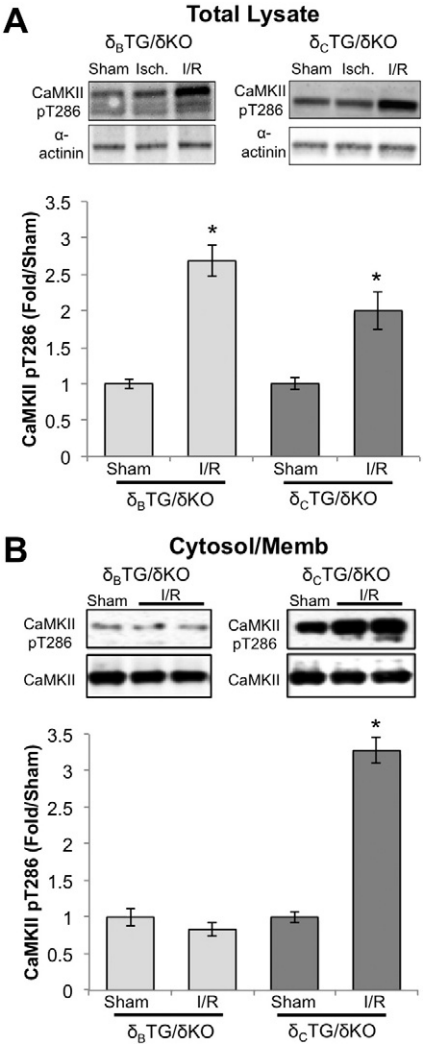


**Fig. 3.** CaMKII$\delta_C$ is selectively activated in a subcellular fraction containing cellular membranes and cytosol. *A*, Lysates from the hearts of $\delta_B$TG/δKO and $\delta_C$TG/δKO mice were subjected to western blot analysis using an antibody specific for autophosphorylated CaMKII. Data are mean ± SEM values from 4 to 6 mice. *B*, Subcellular fractionation of hearts from $\delta_B$TG/δKO and $\delta_C$TG/δKO mice was carried out as described in methods yielding a fraction that includes cytosol and cellular membranes. Data are mean ± SEM values from 4 to 6 mice. *$P < 0.05$ *vs* sham.

To confirm that oxidative stress can lead to selective activation of CaMKII$\delta_C$ and of NF-κB in a cardiomyocyte-autonomous fashion, we infected NRVMs with adenovirus expressing CaMKII$\delta_B$ or CaMKII$\delta_C$. Cells were infected with a range of MOIs of CaMKII$\delta_B$ or $\delta_C$ virus and subsequently treated with 50 μM $H_2O_2$ for 30 min to elicit CaMKII and NF-κB activation. In NRVM whole cell lysates we observed equivalent activation of CaMKII$\delta_B$ and CaMKII$\delta_C$ in response to $H_2O_2$ at all MOIs (Fig. 6A, top panel), as quantitated using an MOI of 50 (Fig. 6A lower panel). In NRVM lysates that were fractionated to enrich for cytosolic and membrane proteins, however, there was significantly greater activation of CaMKII$\delta_C$ following $H_2O_2$ treatment (Fig. 6B). We further determined that expression of CaMKII$\delta_C$, but not CaMKII$\delta_B$, enhanced $H_2O_2$-mediated I kappa B alpha (IκBα) degradation (Fig. 6C) and nuclear p65 accumulation (Fig. 6D). These data, like those obtained in the isolated perfused heart (Figs. 3 and 4) suggest that oxidative stress-induced CaMKII$\delta_C$ activation in the cytosolic/membrane compartment is associated with activation of NF-κB.

To demonstrate that the deleterious effects of CaMKII$\delta_C$ activation seen in the isolated perfused heart were mediated by IKK/NF-κB signaling we blocked IKK activation with the pharmacological inhibitor BMS-345541 (BMS) [21]. Since this drug had not, to our knowledge, been used in the *ex vivo* perfused heart we evaluated its efficacy and determined a dose (5 μmol/L) that sequestered p65 in the cytosol/membrane fraction (Supplemental Fig. 2). Hearts from $\delta_B$TG/δKO, $\delta_C$TG/δKO, WT, and KO mice were then exposed to 5 μmol/L BMS or vehicle prior to and throughout the I/R protocol. Infarct size was significantly reduced by BMS administration in WT and $\delta_C$TG/δKO mice, while the already diminished infarct formation that was observed in $\delta_B$TG/δKO animals was not affected (Fig. 7). CaMKIIδKO animals showed a modest but significant further reduction in infarct size.

In light of the selective increase in TNF-α mRNA in $\delta_C$TG/δKO mice, and evidence that active TNF-α can be produced in and secreted from the isolated perfused heart [22], we asked whether TNF-α mediated the deleterious effects of CaMKII$\delta_C$. For these studies we used etanercept, which blocks the effects of TNF-α by preventing its interaction with TNF-α receptors [23,24]. Etanercept was perfused at 5 μg/mL prior to and throughout the I/R protocol. The results were similar to those obtained with IKK/NF-κB inhibition *i.e.* infarct size was significantly reduced in WT and $\delta_C$TG/δKO mice and was not reduced further in $\delta_B$TG/δKO animals. CaMKIIδKO animals showed modest but significant further reductions in infarct size in the presence of etanercept (Fig. 8). These findings reveal a surprisingly important role for autocrine and/or paracrine TNF-α signaling in the *ex vivo* isolated perfused heart during I/R, demonstrate that this process is regulated by CaMKII$\delta_C$, and establish an essential cardiac-intrinsic role for NF-κB in I/R injury (Fig. 9).

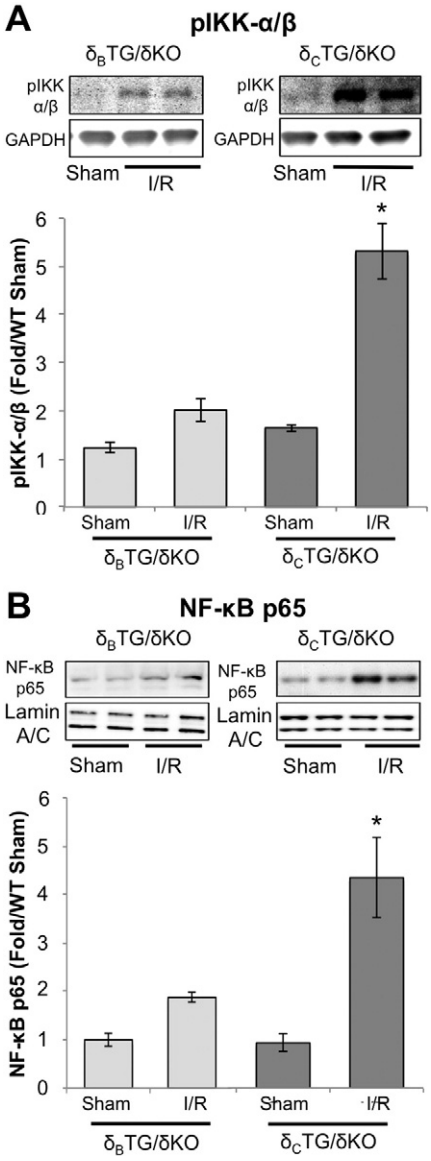


**Fig. 4.** The NF-κB pathway is activated in CaMKIIδ$_C$TG/δKO mice following reperfusion. *A.* Activation of IKK was assessed in δ$_B$TG/δKO and δ$_C$TG/δKO mice using an antibody specific for auto-phosphorylated IKKα/β. *B*, NF-κB p65 accumulation was assessed in nuclear extracts made from hearts of δ$_B$TG/δKO and δ$_C$TG/δKO mice subjected to *ex vivo* I/R. Data are mean ± SEM values from 6 mice. $P < 0.05$ *vs* sham.

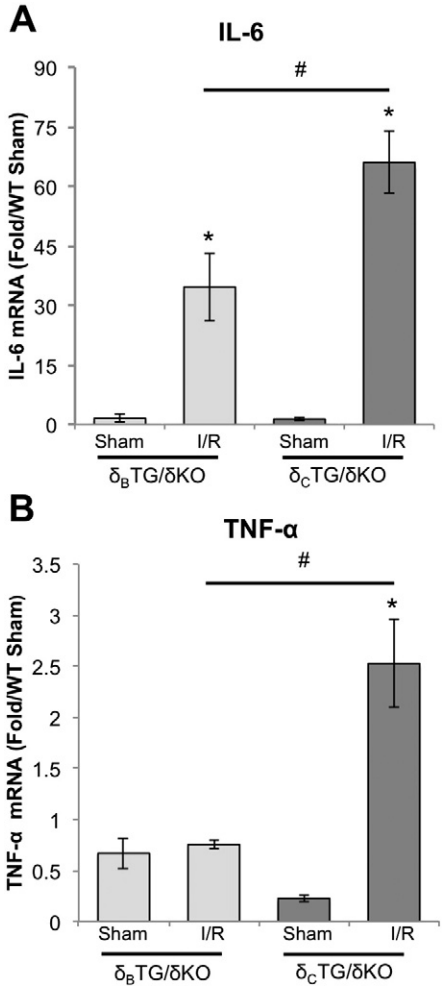


**Fig. 5.** Genes downstream of NF-κB are upregulated in CaMKIIδ$_C$TG/δKO mice after *ex vivo* I/R. NF-κB activation was assessed by measuring mRNA transcripts of genes regulated by NF-κB, *A*, IL-6 *B*, TNF-α. Data are mean ± SEM values from 4 mice. *$P < 0.05$ *vs* sham #$P < 0.05$ *vs* δ$_C$TG/δKO.

## 5. Discussion

Our laboratory previously demonstrated that mice in which CaMKIIδ was selectively deleted from cardiomyocytes exhibited diminished infarct formation in response to *in vivo* I/R [9]. These experiments provided evidence that CaMKIIδ activation in cardiomyocytes mediates the deleterious effects of I/R injury but did not address the question of which subtype(s) of CaMKIIδ were responsible for cardiomyocyte death in response to I/R. Here we characterize and utilize mice in which the CaMKIIδ$_B$ or CaMKIIδ$_C$ isoforms are expressed in the CaMKIIδ KO animals to independently determine the role of each of these isoforms in cardiomyocyte CaMKII signaling. In addition we analyzed I/R damage in the *ex vivo* isolated perfused heart where the role of systemic inflammatory factors is eliminated. Our studies demonstrate that signals that are rapidly and locally generated regulate infarct development in the *ex vivo* heart and are significantly attenuated in the absence of CaMKIIδ. We further show that CaMKIIδ$_C$ re-expression in cardiomyocytes reverses the attenuation of infarct formation seen in CaMKIIδKO mice. In contrast CaMKIIδ$_B$ re-expression further limits infarct development compared to that observed in KO mice.

While total CaMKIIδ expression and maximal activity are somewhat higher in δ$_C$TG/δKO mice (Supplemental Fig. 3 B) the amount of activated ($Ca^{2+}$-autonomous) CaMKII is similar in δ$_C$TG/δKO and δ$_B$TG/δKO animals (Supplemental Fig. 3 A). Thus it is unlikely that differential baseline levels of active CaMKIIδ explain the response of δ$_C$TG/δKO *versus* δ$_B$TG/δKO animals to I/R. There may, however, be a greater propensity for activation of inflammatory responses when CaMKIIδ$_C$ is activated thus we determined whether I/R could lead to selective increases in NF-κB activation in δ$_C$TG/δKO mice. NF-κB has been implicated in *in vivo* I/R damage and myocardial infarction and our earlier studies demonstrated that CaMKIIδ activates NF-κB during *in vivo* I/R [9,25,26]. We previously assumed that *in vivo* I/R triggered systemic responses such as infiltration of non-cardiac inflammatory cells into the heart during the 24-48 h of reperfusion, and that this was a significant contributor to infarct development. Here, however, we demonstrate that CaMKIIδ$_C$ and subsequent NF-κB-mediated responses occur in the isolated perfused heart implicating cardiac-intrinsic signaling in the effects of CaMKIIδ$_C$ on infarct formation.

It is possible that the deleterious effect of CaMKIIδ$_C$ is initiated by induction of cardiomyocyte cell death and release of factors from necrotic cells that induce inflammatory signaling [27,28]. Our previous work showed, however, that cyclosporine-A (CsA) treatment did not block NF-κB activation in response to *in vivo* I/R, indicating that this response

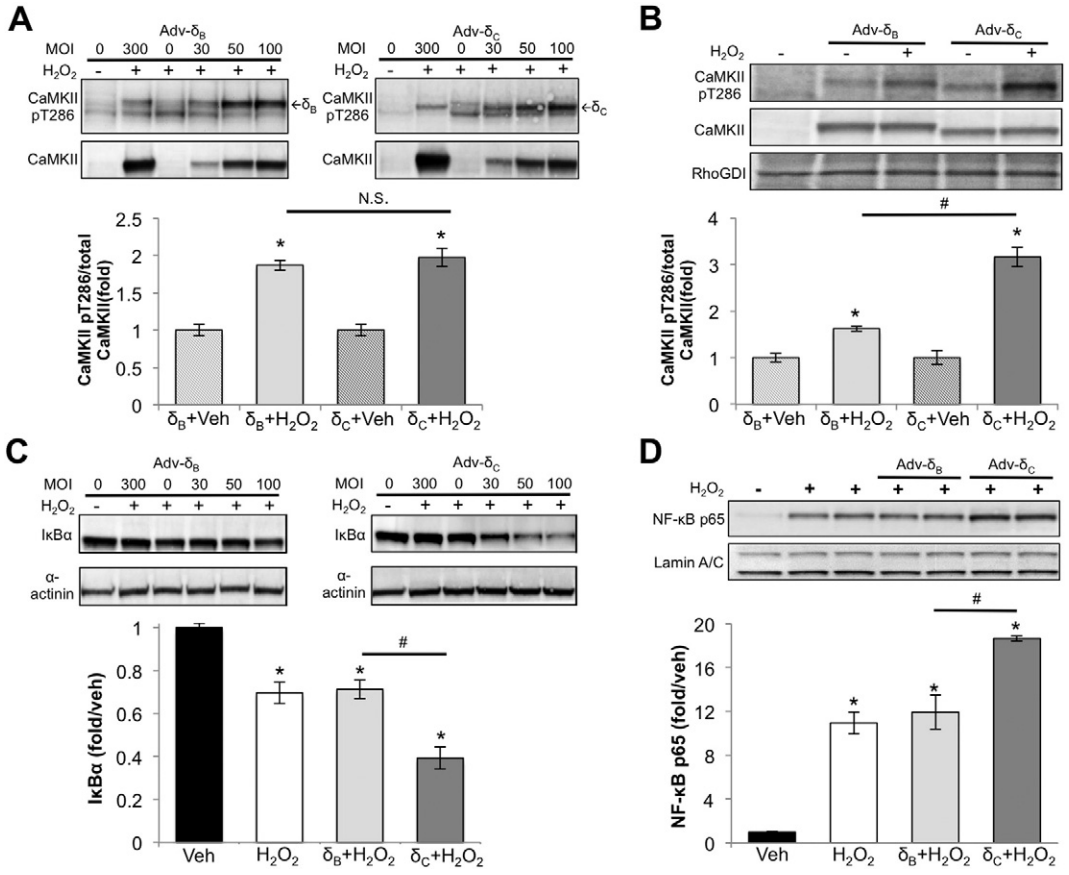


**Fig. 6.** Selective CaMKII$\delta_C$ and NF-κB activation in NRVM treated with 50 μM $H_2O_2$ for 30 m *A*) CaMKII activation measured in NRVM infected with adenovirus, MOI of 50 was quantified. *B*) CaMKII activation measured in a cytosolic/membrane fraction isolated from NRVM expressing $\delta_B$ or $\delta_C$ (MOI 50). *C*) IκBα degradation assessed in NRVM infected with adenovirus. MOI of 50 was quantified. *D*) NF-κB p65 measured in nuclear extracts from NRVM expressing $\delta_B$ or $\delta_C$ (MOI 50). All data are mean ± SEM $n = 3$ $^*P < 0.05$ *vs* Veh #$P < 0.05$ *vs* $\delta_C$.

was independent of necrosis [9] and we confirmed this in the current *ex vivo* study (Supplemental Fig. 4). Accordingly we suggest that there is a direct effect of CaMKII on NF-κB activation. This conclusion is further supported by our current data showing that acute activation of CaMKII$\delta_C$ with $H_2O_2$ elicits NF-κB activation in NRVMs and by our previous demonstration that adenoviral expression of activated CaMKII$\delta_C$ in NRVMs induces IKK activation [9].

Strikingly, we show here that NF-κB activation in response to I/R is enhanced by cardiac expression of CaMKII$\delta_C$ but not CaMKII$\delta_B$. This is consistent with the finding that phosphorylation of the upstream kinase IKK is increased by I/R in $\delta_C$TG/δKO but not in $\delta_B$TG/δKO animals. Furthermore, we demonstrate that I/R increases activation of CaMKII$\delta_C$ but not of CaMKII$\delta_B$ in the cytosol/membrane, where IKK is localized. We previously reported that despite its nuclear localization sequence [29] CaMKII$\delta_B$ is not strictly confined to the nucleus [14] but is in fact present in the cytosol/membrane compartment (Fig. 3B). It remains unclear why there is selective activation of CaMKII$\delta_C$ and not CaMKII$\delta_B$ in this location. Nevertheless our observations in NRVMs confirm that there is differential activation of equivalently expressed levels of CaMKII$\delta_B$ and $\delta_C$ by oxidative stress and further indicate an association between cytosolic CaMKII$\delta_C$ autophosphorylation and subsequent IκBα degradation and nuclear accumulation of p65. Notably another recent report confirmed the ability of CaMKIIδ subtypes to undergo differential posttranslational modification during reperfusion [30].

Previous studies using pharmacological inhibitors to demonstrate involvement of CaMKIIδ in *ex vivo* I/R damage linked the effects of CaMKII to phosphorylation of PLN and RyR2 and subsequent $Ca^{2+}$ dysregulation [31], supporting the hypothesis that rapid phosphorylation of CaMKII targets was a driver of myocardial injury. Indeed, we originally considered it unlikely that transcriptional regulation by CaMKII$\delta_C$-mediated activation of NF-κB could play a significant role in determining cell viability over the course of only one hour of reperfusion. Surprisingly, however, we found that inhibition of either IKK or TNF-α had a profound effect on infarct formation not only in $\delta_C$TG/δKO but also in WT mouse hearts subjected to 25 min *ex vivo* ischemia and 60 min reperfusion.

Recent studies have shown that receptor-interacting protein kinase 3 (RIP3) plays a role in TNF-α-mediated cell death through formation of the necrosome [32]. RIP3 phosphorylates and activates CaMKII during I/R [33] accordingly TNF-α could participate in a deleterious positive feedback loop leading to sustained CaMKII$\delta_C$ and NF-κB activation. The experiments presented in this study clearly indicate that TNF-α signaling and myocardial NF-κB activation are mechanisms by which CaMKII$\delta_C$ elicits infarct formation in the isolated perfused heart. This signaling would likely be further enhanced and sustained by $Ca^{2+}$ dysregulation resulting from CaMKII-mediated phosphorylation of SR substrates and increases in reactive oxygen species (ROS) during I/R [19,34,35].

Paradoxically, some reports indicate that NF-κB may be protective in I/R injury [36,37] while others demonstrate a deleterious role for NF-κB activation [25,38]. Importantly, these studies differ in how I/R is elicited and how NF-κB is inhibited, thus resolution of these conflicting conclusions remains elusive. In the current study we have demonstrated that CaMKII$\delta_C$ expressed in cardiomyocytes mediates NF-κB activation, TNF-α induction and infarct development during I/R and that these events occur rapidly and in the absence of systemic inflammatory factors. Our data contribute to the understanding of the dichotomous effects of NF-κB by clearly demonstrating an adverse cardiomyocyte-autonomous role of NF-κB activation in I/R injury.

While we demonstrate that cardiac TNF-α expression during I/R is mediated by CaMKII$\delta_C$ activation in cardiomyocytes, we do not know

the extent to which TNF-α is formed in and secreted from these cells. Cardiac-resident macrophages and other non-myocytes are potential sources of TNF-α and could thus act in an autocrine or paracrine fashion to affect cardiomyocyte survival. The extent to which CaMKIIδ$_C$ activation in myocytes sends signals to other cells is not clear, but our studies demonstrate that the cardiomyocyte initiates signals though CaMKIIδ that lead to upregulation of TNF-α. TNF-α inhibitors such as etanercept have been used to inhibit TNF-α signaling in patients with various autoimmune disorders [39]. Effects of TNF-α inhibitors on cardiovascular disease outcomes have also been evaluated with uncertain results that could in part be due to systemic responses to sustained antibody administration [28]. Such detrimental effects would not be expected to occur during short-term treatment thus use of TNF-α inhibitors could be of value if employed at the onset of reperfusion following primary percutaneous intervention for myocardial infarction.

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

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2017.01.002.

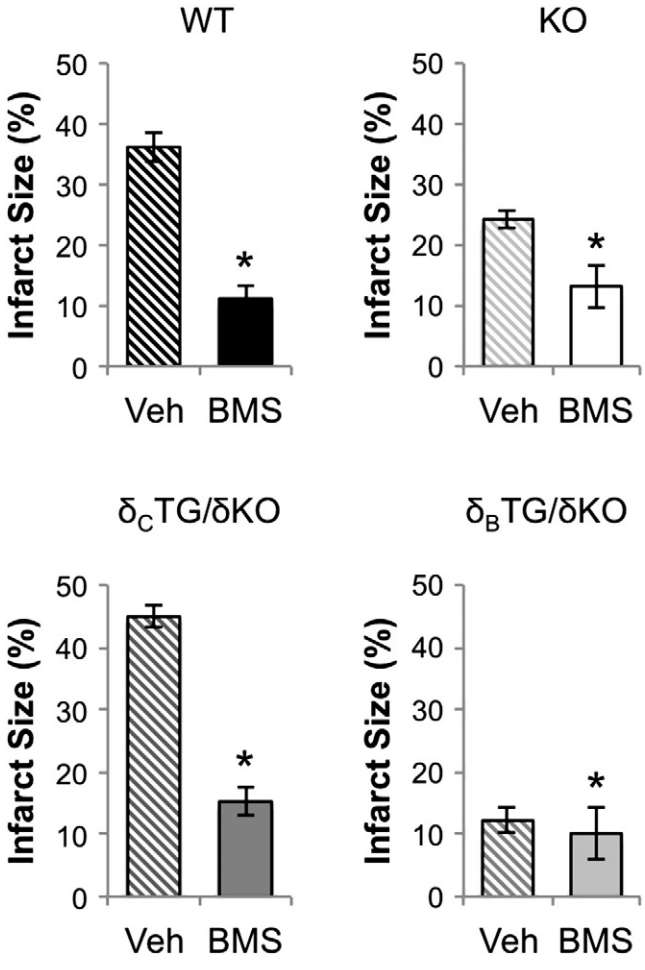


**Fig. 7.** Inhibition of IKK ameliorates I/R damage. Vehicle and BMS-345541 (5μmol/L) were perfused into hearts from WT, KO, δ$_B$TG/δKO, and δ$_C$TG/δKO animals. Infarct size was measured *via* TTC staining following 1hr of reperfusion. Data are mean ± SEM values from 4 to 8 mice. *$P < 0.05$ *vs* veh.

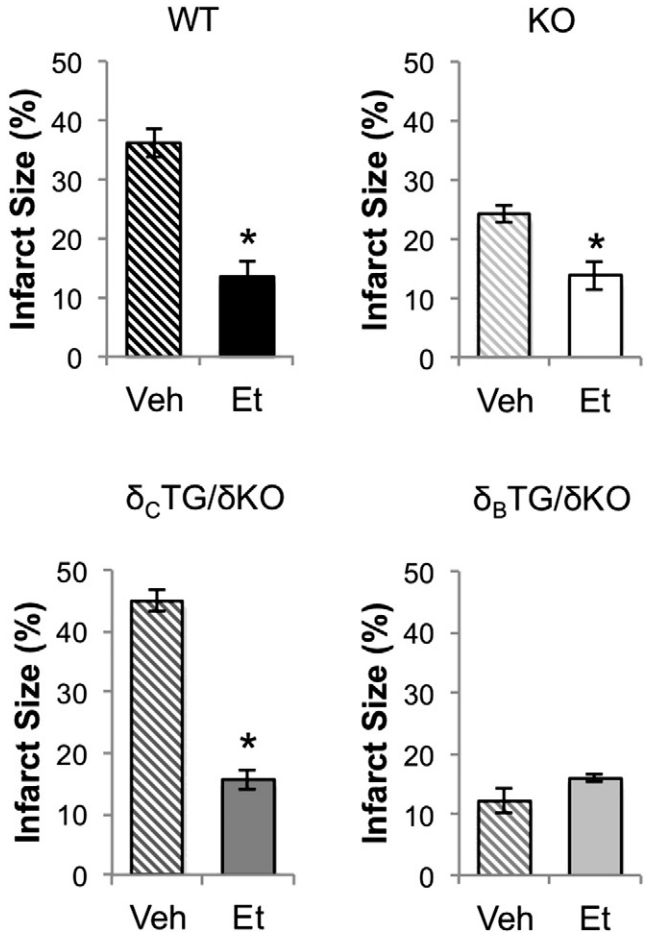


**Fig. 8.** Inhibition of TNF-α ameliorates I/R damage. Vehicle or etanercept (5 μg/mL) were perfused into hearts from WT, KO. δ$_B$TG/δKO, and δ$_C$TG/δKO animals Infarct size was measured *via* TTC staining following 1hr of reperfusion. Data are mean ± SEM values from 4 to 8 mice. *$P < 0.05$ *vs* veh.

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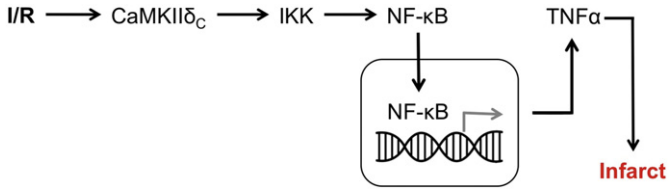


**Fig. 9.** Summary of findings. NF-κB nuclear translocation and subsequent TNF-α upregulation during I/R is mediated by cytosolic CaMKIIδ$_C$ activation.

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## Disclosures

None.

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