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Cardioprotection by CaMKII- δ B Is Mediated by Phosphorylation of HSF1 and Subsequent Expression of Inducible HSP70

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Abstract

Rationale—Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional kinase involved in vital cellular processes such as Ca²⁺ handling and cell fate regulation. In mammalian heart, two primary CaMKII isoforms, δ B and δ C, localize in nuclear and cytosolic compartments, respectively. Although previous studies have established an essential role of CaMKII- δ C in cardiomyocyte apoptosis, the functional role of the more abundant isoform, CaMKII- δ B, remains elusive.

Objective—Here we determined the potential role of CaMKII- δ B in regulating cardiomyocyte viability and explored the underlying mechanism.

Methods and Results—In cultured neonatal rat cardiomyocytes, the expression of CaMKII- δ B and CaMKII- δ C was inversely regulated in response to H₂O₂-induced oxidative stress with a profound reduction of the former and an increase of the later. Similarly, *in vivo* ischemia/reperfusion (IR) led to an opposite regulation of these CaMKII isoforms in a rat myocardial IR model. Notably, overexpression of CaMKII- δ B protected cardiomyocytes against oxidative stress-, hypoxia- and angiotensin II-induced apoptosis, whereas overexpression of its cytosolic counterpart promoted apoptosis. Using cDNA microarray, real time-PCR and Western blotting, we demonstrated that overexpression of CaMKII- δ B but not CaMKII- δ C elevated expression of heat shock protein 70 (HSP70) family members, including inducible HSP70 (iHSP70) and its homologous (Hst70). Moreover, overexpression of CaMKII- δ B led to phosphorylation and activation of heat shock factor 1 (HSF1), the primary transcription factor responsible for HSP70 gene regulation. Importantly, gene silencing of iHSP70, but not Hst70, abolished CaMKII- δ B-mediated protective effect, indicating that only iHSP70 was required for CaMKII- δ B elicited anti-apoptotic signaling.

Conclusions—We conclude that cardiac CaMKII- δ B and CaMKII- δ C were inversely regulated in response to oxidative stress and IR injury, and that in contrast to CaMKII- δ C, CaMKII- δ B serves as a potent suppressor of cardiomyocyte apoptosis triggered by multiple death-inducing stimuli via phosphorylation of HSF1 and subsequent induction of iHSP70, marking both CaMKII- δ isoforms as promising therapeutic targets for the treatment of ischemic heart disease.

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Keywords

CaMKII isoforms; CaMKII- δ B; oxidative stress; hypoxia; cardiomyocyte apoptosis; iHSP70; HSF1

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is a ubiquitous and multifunctional serine/threonine protein kinase family, which consists of 30 distinct members encoded by four different genes (α , β , δ , and γ). The δ isoform of CaMKII is predominantly expressed in the heart of many mammalian species, including rat, mouse and human¹⁻⁴. Two primary splicing variants of the δ isoform, CaMKII- δ B and CaMKII- δ C, have been cloned from rat heart¹. The only difference between these isoforms is the additional 11-amino acids conferring a nuclear targeting signal of CaMKII- δ B⁵. As a result, CaMKII- δ B and CaMKII- δ C localize to the nucleus and the cytoplasm, respectively, although they share many common biochemical properties⁵.

CaMKII is activated upon the binding of Ca²⁺ and calmodulin complex^{6, 7}. In addition to the widely-accepted Ca²⁺/calmodulin-mediated activation, recent studies have demonstrated that CaMKII can be activated by reactive oxygen species (ROS)-induced oxidation in a Ca²⁺-independent fashion⁸. Activation of CaMKII leads to phosphorylation of a panel of target proteins involved in multiple important physiological and pathological processes from Ca²⁺ handling, muscle contraction to memory encoding⁹⁻¹¹.

With respect to isoform-specific functions in the heart, most studies over the past decade have been focused on the role of CaMKII- δ C in regulating cardiac excitation-contraction coupling¹²⁻¹⁴ and maladaptive cardiac remodeling¹⁵. In addition, recent studies have shown that CaMKII- δ C is a common intermediate of cardiac cell apoptosis induced by diverse death-inducing stimuli, including excessive β_1 -adrenergic receptor (β_1 -AR) stimulation, H₂O₂, intracellular high Ca²⁺, and acidosis¹⁶⁻¹⁸. Despite the higher abundance of CaMKII- δ B, it has received far less attention than its cytosolic counterpart. The functional role of CaMKII- δ B in the heart remains largely unknown, although emerging evidence suggests that overexpression of CaMKII- δ B induces cardiac hypertrophy^{19, 20}. In the current study, we demonstrate that the expression of the major cardiac CaMKII isoforms are inversely regulated in response to oxidative stress and ischemia/reperfusion (IR) injury, and that enhanced CaMKII- δ B signaling potently protects cardiomyocytes against oxidative stress-, hypoxia-, and angiotensin II (Ang II)-induced apoptosis, while activation of CaMKII- δ C is apoptotic. The anti-apoptotic effect is mediated by CaMKII- δ B-induced phosphorylation of an important transcriptional factor, heat shock factor 1 (HSF1), which positively regulates the expression of inducible heat shock protein70 (iHSP70, also known as HSP72). These findings not only define a novel cardiac protective function of CaMKII- δ B, but also imply that CaMKII- δ B downregulation or malfunction may be a crucial pathogenic element and a potential therapeutic target for various forms of heart disease, particularly ischemic heart disease.

METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich. An expanded Methods section is available in the online data supplement at <http://circres.ahajournals.org>.

Isolation, Culture and Adenoviral Infection of Rat Ventricular Myocytes

Neonatal or adult rat ventricular myocytes were isolated, cultured and infected as previously described^{16, 21}. See the online data supplement for further details.

Western Blotting Analysis, and Confocal Immunocytochemical Imaging of Hemagglutinin (HA)-tagged CaMKII- δ B or CaMKII- δ C

Separation of cytosolic and nuclear protein was achieved with a Nuclear/Cytosolic Fractionation Kit (Biovision Research Products, CA, USA) following the manufacturer's instructions. Western blot to assay the abundance of CaMKII- δ in cytosolic and nuclear extracts was performed with an anti-CaMKII- δ antibody (Santa Cruz Biotechnology, Inc., sc-5392), as previously described¹⁷. Confocal immunocytochemical imaging was performed as previously described¹⁶. See the online data supplement for further details.

Cell Viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or DNA laddering/Hoechst staining was used as cell viability or apoptosis index, respectively, as described previously²¹. See the online data supplement for further details.

Small Interfering RNA (siRNA)

Cardiomyocytes were transfected with siRNA targeted specifically to iHSP70 or Hst70 by Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer's instructions. See the online data supplement for further details.

Statistical Analysis

Data are expressed as the mean \pm S.E.. Statistical comparisons used one-way analysis of variance, followed by Bonferroni's procedure for multiple-group comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Oxidative Stress Oppositely Regulates the Expression Profile of CaMKII- δ B and CaMKII- δ C in Cultured Neonatal Rat Cardiomyocytes

To determine the potential functional role of CaMKII- δ B in regulating cardiomyocyte viability, we first examined the expression of the major cardiac CaMKII isoforms, CaMKII- δ B and CaMKII- δ C, in response to oxidative stress. H₂O₂ treatment elevated CaMKII- δ C gene expression in a concentration- and time-dependent manner (Figure 1 A&B). In contrast, CaMKII- δ B gene expression was profoundly suppressed in response to H₂O₂ treatment (Figure 1 A&B). To determine whether these CaMKII isoforms were also oppositely regulated at their protein levels by oxidative stress, we fractionalized cardiomyocyte proteins and detected CaMKII- δ B and CaMKII- δ C abundance by assaying CaMKII- δ in the nuclear and the cytosolic fractions, respectively, using the same antibody reacting with both CaMKII- δ isoforms. CaMKII- δ B protein abundance was rapidly and profoundly reduced by half at 4 h after H₂O₂ (200 μ M) treatment and reached the minimal level at 24 h after treatment, whereas the protein level of CaMKII- δ C was significantly elevated after H₂O₂ exposure (Figure 1C).

IR Oppositely Regulates CaMKII- δ B and CaMKII- δ C Expression *In Vivo*

To investigate whether CaMKII- δ B and CaMKII- δ C were oppositely regulated *in vivo* during cardiac IR injury, we utilized a rat myocardial IR model. The experimental protocol and cardiac damage readouts, including myocardial infarction and lactate dehydrogenase (LDH) release, were illustrated in Online Figure I (online data supplements). Importantly, IR led to a marked increase in CaMKII- δ C and a decrease in CaMKII- δ B expression at both mRNA and protein levels (Figure 1 D-E). These results indicate there is an inverse regulation of the expression of CaMKII- δ B and CaMKII- δ C by oxidative stress or IR injury *in vivo* as well as in cultured cardiomyocytes, highlighting that these CaMKII isoforms may elicit distinctly different, even opposing functional roles in regulating cardiomyocyte viability.

Overexpression of CaMKII- δ B Promotes Cardiomyocyte Viability

To test the above hypothesis, we investigated whether activation of the nuclear isoform, CaMKII- δ B, regulates cardiac cell viability and, if so, whether the effect of CaMKII- δ B differs from that of CaMKII- δ C. Using adenoviral gene transfer, we overexpressed either CaMKII- δ B or CaMKII- δ C in cultured neonatal or adult rat cardiomyocytes in the presence or absence of H₂O₂ (200 μ M). Overexpressed CaMKII- δ B was enriched in the nuclear compartment while CaMKII- δ C in the cytosol in neonatal cardiomyocytes (Figure 2A), as is the case in adult rodent cardiac myocytes¹⁶. Expression levels and phosphorylation status of both CaMKII isoforms were elevated in a titer-dependent manner in neonatal myocytes infected with Adv-CaMKII- δ B or Adv-CaMKII- δ C for 24 h (Figure 2B). H₂O₂ exposure triggered robust cardiomyocyte apoptosis, as manifested by DNA laddering (Figure 2 C&D) and an increase in Hoechst staining-positive cells (Figure 2E) compared with non-treated cells. Remarkably, overexpression of CaMKII- δ B, but not CaMKII- δ C or β -gal, protected both neonatal and adult rat cardiac myocytes from H₂O₂-mediated apoptosis (Figure 2 C&D) as well as necrosis (Online Figure II, online data supplements). H₂O₂-induced DNA fragmentation assayed by DNA laddering (Figure 2 C&D) and apoptotic nuclear morphological changes visualized by Hoechst staining (Figure 2E) were markedly blunted in cells infected by Adv-CaMKII- δ B. In contrast, overexpression CaMKII- δ C promoted apoptosis in cultured neonatal cardiac myocytes even in the absence of H₂O₂ treatment, and overtly enhanced apoptosis induced by H₂O₂ (100 μ M) (Figure 2 E). CaMKII- δ C-induced apoptosis was abolished by overexpression of CaMKII- δ B (Online Figure III, online data supplements).

Next, we determined whether CaMKII- δ B can protect cardiomyocytes from other death-inducing stimuli, in addition to oxidative stress. Overexpression of CaMKII- δ B abolished hypoxia- and Ang II-induced myocyte apoptosis (Figure 2 F-G, respectively). These results provided the first documentation that activation of the most abundant cardiac CaMKII isoform, CaMKII- δ B, potently protects cardiomyocytes against apoptosis triggered by multiple death-inducing stimuli, whereas its cytosolic counterpart promotes apoptosis.

An Essential Role of iHSP70 in CaMKII- δ B-Mediated Cardiomyocyte Protection

To delineate the mechanism underlying the protective effect of CaMKII- δ B, we examined possible differential gene expression profiles with cDNA microarray analysis in neonatal cardiomyocytes infected with Adv-CaMKII- δ B, Adv-CaMKII- δ C, or Adv- β -gal (all at 20 m.o.i for 36 h). The expression levels of 174 genes were significantly altered in neonatal cardiomyocytes infected with Adv-CaMKII- δ B relative to those in cells infected with Adv- β -gal (>1.5-fold or < 0.5-fold, n = 3). Among them, 17 genes were specifically upregulated or downregulated by overexpression of CaMKII- δ B but not CaMKII- δ C (>1.5 fold or <0.5 fold, n = 3) (Online Table I and Online Figure IV, online data supplements), although the majority of the 174 genes were similarly regulated by both CaMKII isoforms. Importantly, among the 17 differentially regulated genes, we identified an important cell protective factor, iHSP70 (also known as HSP72), which was selectively upregulated in response to overexpression of CaMKII- δ B but not CaMKII- δ C. The cDNA microarray analysis revealed a 1.7-fold increase in iHSP70 gene expression in cells infected with Adv-CaMKII- δ B but not Adv-CaMKII- δ C relative to those infected by Adv- β -gal (Figure 3A). Using real-time PCR, we validated the CaMKII- δ B-dependent elevation of iHSP70 gene expression at either 36 h or 48 h after adenoviral infection (Figure 3B). Likewise, iHSP70 protein abundance was also augmented by 2.4-folds only in cells infected with Adv-CaMKII- δ B (m.o.i. 20 for 48 h) (Figure 3C).

To determine whether the elevation in iHSP70 expression is causatively linked to CaMKII- δ B-induced anti-apoptotic effect, we utilized siRNA-mediated iHSP70 gene silencing. siRNAs (including siRNA1 and siRNA2) effectively reduced the expression of iHSP70 at mRNA (Figure 4A) and protein levels (Figure 4B). Notably, iHSP70 gene silencing substantially

abolished CaMKII- δ B-mediated anti-apoptotic effect, as assayed by DNA laddering in cardiomyocytes treated with either siRNA1 or siRNA2 of iHSP70, regardless of CaMKII- δ B overexpression (Figure 4C). Moreover, MTT assay further revealed that overexpression of CaMKII- δ B could not protect cardiomyocytes from H₂O₂-induced cell death in the presence of iHSP70 siRNA1 or siRNA2 (Figure 4D). These results provide strong evidence to indicate that upregulation of iHSP70 is essentially involved in CaMKII- δ B-elicited anti-apoptotic signaling.

Interestingly, another closely related member of the HSP70 family, namely Hst70 in rats (but called HSP70.2 in mouse), was also significantly increased by 1.8-fold in cells overexpressing CaMKII- δ B but not in those overexpressing CaMKII- δ C (Figure 3A and Online Table I, online data supplements). However, gene silencing of Hst70 had no detectable effect on CaMKII- δ B-induced protection. Specifically, two out of three tested siRNA sequences (Hst70 siRNA1 and 3) suppressed Hst70 mRNA level by 60-70 % (Figure 5A), but could not abolish the protective effect of CaMKII- δ B assayed by DNA laddering (Figure 5B) and MTT (Figure 5C), indicating that Hst70, unlike HSP72, is not involved in CaMKII- δ B-mediated anti-apoptotic signaling.

CaMKII- δ B Overexpression Increases Phosphorylation of Nuclear HSF1 at Ser230

To investigate the mechanism underlying CaMKII- δ B-mediated upregulation of iHSP70, we analyzed the expression and phosphorylation status of HSF1, the transcription factor primarily responsible for the regulation of iHSP70 expression. Immunofluorescent imaging revealed that CaMKII- δ B and HSF1 were co-localized at the nuclear compartment in cardiomyocytes infected with Adv-CaMKII- δ B (Figure 6A). Using a site-specific antibody reacting with phosphorylated HSF1 at Ser230, which has been shown to positively contribute to the transcriptional activity of HSF1²², we found that overexpression of CaMKII- δ B but not CaMKII- δ C or β -gal elevated phosphorylation level of HSF1 at Ser230 relative to the control group (Figure 6B). In contrast, overexpression of CaMKII- δ B had no detectable effect on phosphorylation of HSF1 at Ser303, a constitutively phosphorylated residue, indicating that CaMKII- δ B selectively phosphorylates HSF1 at Ser230 (Figure 6B). It is noteworthy that H₂O₂ (200 μ M for 15 min) treatment transiently increased CaMKII- δ B mediated phosphorylation of HSF1-Ser230 (Figure 6C) and HSF1 nuclear translocation (Online Figure V, online data supplements).

DISCUSSION

Cardiac myocyte apoptosis plays a pivotal role in the development of a variety forms of heart disease, including ischemic heart disease, congestive heart failure, and acute myocardial infarction^{23, 24}. ROS has been defined as a major contributor in triggering cardiomyocyte apoptosis during myocardial ischemia/reperfusion injury, dilated cardiomyopathy and the progression of heart failure²⁵. Recent studies have established a link between oxidative stress and activation of CaMKII and defined an essential role of CaMKII signaling in oxidative stress-triggered cardiomyocyte apoptosis^{8, 17}. However, isoform-specific expression regulation and function of cardiac CaMKII- δ B and CaMKII- δ C in response to oxidative stress, IR injury, or hypoxia have not been well characterized until now. Given their distinct intracellular distributions, these cardiac CaMKII isoforms may be differentially regulated and exhibit different actions. Indeed, in this study, we have revealed following important differences between these CaMKII- δ isoforms in their responses to oxidative stress or IR injury and their functional roles in regulating cardiac muscle cell viability. First, the expression profile of CaMKII- δ B and CaMKII- δ C is inversely regulated in cardiomyocytes subjected to oxidative stress with a downregulation of CaMKII- δ B and an upregulation of CaMKII- δ C. Similar opposite regulation of these cardiac CaMKII- δ isoforms is induced by myocardial IR injury

in vivo. Second, overexpression of CaMKII- δ B markedly attenuates H₂O₂-, hypoxia-, and AngII-induced apoptotic cell death, whereas overexpression of CaMKII- δ C promotes cardiomyocyte apoptosis. Moreover, overexpression of CaMKII- δ B, but not CaMKII- δ C, selectively elevates phosphorylation of HSF1 at Ser230, resulting in increased expression of the major inducible HSP70, iHSP70, that is obligated to CaMKII- δ B-induced cardiomyocyte protection. These present findings support the perception that the nuclear-located isoform, CaMKII- δ B, constitutes an important cardiac protective factor against multiple insulting stimuli-induced myocardial damage, and that a reduction in this beneficial isoform in conjunction with a simultaneous increase in the detrimental isoform, CaMKII- δ C, is a potential cause factor of various cardiac disorders, including cardiac myocyte apoptosis, IR injury, cardiac arrhythmia, and heart failure²⁶⁻²⁹.

Although the present findings underscore a potentially important anti-apoptotic effect of the nuclear-located isoform, CaMKII- δ B, it is noteworthy that overexpression of CaMKII- δ B activates hypertrophic gene expression and produces hypertrophy in cultured cardiomyocytes³⁰. Similarly, transgenic mice with cardiac-specific overexpression of the nuclear-targeted cardiac CaMKII-isoform develop cardiac hypertrophy, cardiomyocyte enlargement and increases in hypertrophic gene expression²⁰. Thus, exaggerated upregulation of either cardiac CaMKII isoform or an imbalance between these two major isoforms might cause pathological consequences in the heart.

Isoform-Specific Function and Regulation of Cardiac CaMKII- δ B and CaMKII- δ C

Differences between these CaMKII- δ isoforms are manifested by the distinct phenotypes of transgenic mouse models with cardiac-specific overexpression of CaMKII- δ B or CaMKII- δ C. Although both transgenic models can develop cardiac hypertrophy, CaMKII- δ C transgenic mice display much more accelerated cardiac functional deterioration and shortened life span as compared to CaMKII- δ B transgenic mice even with a matched increase in their total cellular CaMKII activity^{15, 20}. Additionally, transgenic overexpression of the cytosolic isoform, CaMKII- δ C, leads to hyper-phosphorylation of substrates involved in cardiac excitation-contraction coupling such as ryanodine receptor (RyR) and phospholamban (PLB), resulting in increased SR Ca²⁺ sparks and decreased SR Ca²⁺ load^{12, 15}, whereas none of these parameters are changed in age-matched transgenic mice with the nuclear targeted CaMKII- δ B²⁰. The isoform-specific phenotypes of CaMKII- δ B and CaMKII- δ C transgenic mouse models can be explained, at least in part, by our previous notion that overexpression of CaMKII- δ C, instead of CaMKII- δ B, exaggerates the adult mouse cardiomyocyte apoptotic death induced by β_1 -AR stimulation and relays apoptotic signal for multiple death-inducing stimuli^{16, 17}. Here, we have further demonstrated that CaMKII- δ B, opposite to CaMKII- δ C, protects cardiomyocytes against apoptosis induced by multiple insulting stimuli such as oxidative stress, hypoxia and AngII stimulation. It is also noteworthy that in response to oxidative stress and IR injury, expression of CaMKII- δ B and CaMKII- δ C are inversely regulated at gene and protein levels. The downregulation of the protective factor, CaMKII- δ B, as well as the upregulation of the deleterious factor, CaMKII- δ C, are expected to contribute to oxidative stress- or IR-induced cell injury and cell death. Taken together, there are distinct differences between these cardiac CaMKII isoforms in their function and regulation under various physiological and pathological circumstances.

Cardioprotection by CaMKII- δ B Is Mediated by HSF1 Phosphorylation and Subsequent Upregulation of iHSP70

HSP belong to an important family of endogenous protective proteins that rapidly respond to a wide variety of stress stimuli, such as heat shock, hypoxia, ischemia, increased ROS, and inflammation. Among various members of HSP family, HSP70 has been implicated as cardiac protective³¹⁻³³. The HSP70 family consists of, at least, eight highly homologous members,

including constitutively expressed (or cognate) and highly inducible proteins that differ from each other by the intracellular localization and expression pattern³⁴. In particular, Hst70 (encoded by Hspa2 gene) is expressed at high levels in testis and at considerably lower or undetectable levels in other tissues^{35, 36}. In contrast, iHSP70 (also named HSP72, encoded by Hspa1b gene) is expressed at low or undetectable levels in most unstressed normal cells and tissues, but its expression is rapidly induced by multiple physical and chemical stresses, and therefore it is often called the major stress-inducible HSP70. Nevertheless, both iHSP70 and Hst70 can be induced under diverse cell stress conditions^{37, 38}, and have been implicated in promoting cell survival^{39, 40}.

In searching for potential mechanisms responsible for the protective effect of CaMKII- δ B, we have found that both iHSP70 and Hst70 are specifically upregulated by overexpression of CaMKII- δ B but not its cytosolic counterpart. Importantly, gene silencing of iHSP70 blocks the protective effect of CaMKII- δ B, establishing an essential role of iHSP70 in relaying CaMKII- δ B anti-apoptotic signaling. In contrast, gene silencing of the closely related HSP70 family member, Hst70, has no detectable impact on CaMKII- δ B-mediated protection. Thus, iHSP70 but not its homologous Hst70 plays an essential role in delivering CaMKII- δ B activated anti-apoptotic signaling.

HSF1 is the transcription factor responsible for stress-induced expression of HSP70. In unstressed cells, HSF1 is present in both the cytoplasm and nucleus in a monomeric form that has no DNA binding activity. In response to heat shock and other stress stimuli, HSF1 assembles into a trimer, accumulates within the nucleus and binds with the proximal promoter heat shock element (HSE) on the HSP70 gene to induce the transcription of HSP70⁴¹⁻⁴³. Previous studies have shown that hyper-phosphorylation of HSF1 is required for the transcriptional competence of the factor⁴⁴⁻⁴⁶. There are two kinds of phosphorylation sites on HSF1, i.e. inducible residue (Ser 230), which positively contributes to the transcriptional competence of HSF1²², and constitutive residues (Ser 303, Ser 307 and Ser363), which repress the transcriptional activity of HSF1^{47, 48}.

In the present study, we have demonstrated that CaMKII- δ B and HSF1 are co-localized in the nuclear compartment and that phosphorylation of HSF1 at Ser230, but not a constitutive residue Ser303, is clearly elevated in myocytes infected with Adv-CaMKII- δ B in the presence or absence of H₂O₂ treatment (Online Figure V, online data supplements). Our data indicate that CaMKII- δ B specifically phosphorylates the inducible residue of HSF1-Ser230, and subsequently enhances its transcriptional activity, leading to increased expression of iHSP70 which is obligated to CaMKII- δ B-mediated cardioprotection.

In summary, we have demonstrated, for the first time, oxidative stress and IR injury leads to a downregulation of CaMKII- δ B and an upregulation of CaMKII- δ C, and that the two major cardiac CaMKII isoforms exhibit opposing effects on cardiomyocyte viability with CaMKII- δ B anti-apoptotic and CaMKII- δ C pro-apoptotic (Figure 7). Phosphorylation of HSF1 and subsequent induction of iHSP70 are essentially involved in CaMKII- δ B-mediated cell survival signaling (Figure 7). These findings mark both cardiac CaMKII isoforms as promising therapeutic targets for preventing or alleviating cardiomyocyte loss caused by myocardial infarction, IR injury, heart failure, and many other etiologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

β_1 -AR	β_1 -adrenergic receptor
Adv	Adenovirus
AngII	Angiotensin II
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
HA	Hemagglutinin
HSE	Heat shock element
HSF1	Heat shock factor 1
Hst70	Testis-specific heat shock protein 70
iHSP70	Inducible heat shock protein 70
IR	Ischemia/reperfusion
LDH	Lactate dehydrogenase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
PLB	Phospholamban
ROS	Reactive oxygen species
RyR	Ryanodine receptor
siRNA	Small interfering RNA

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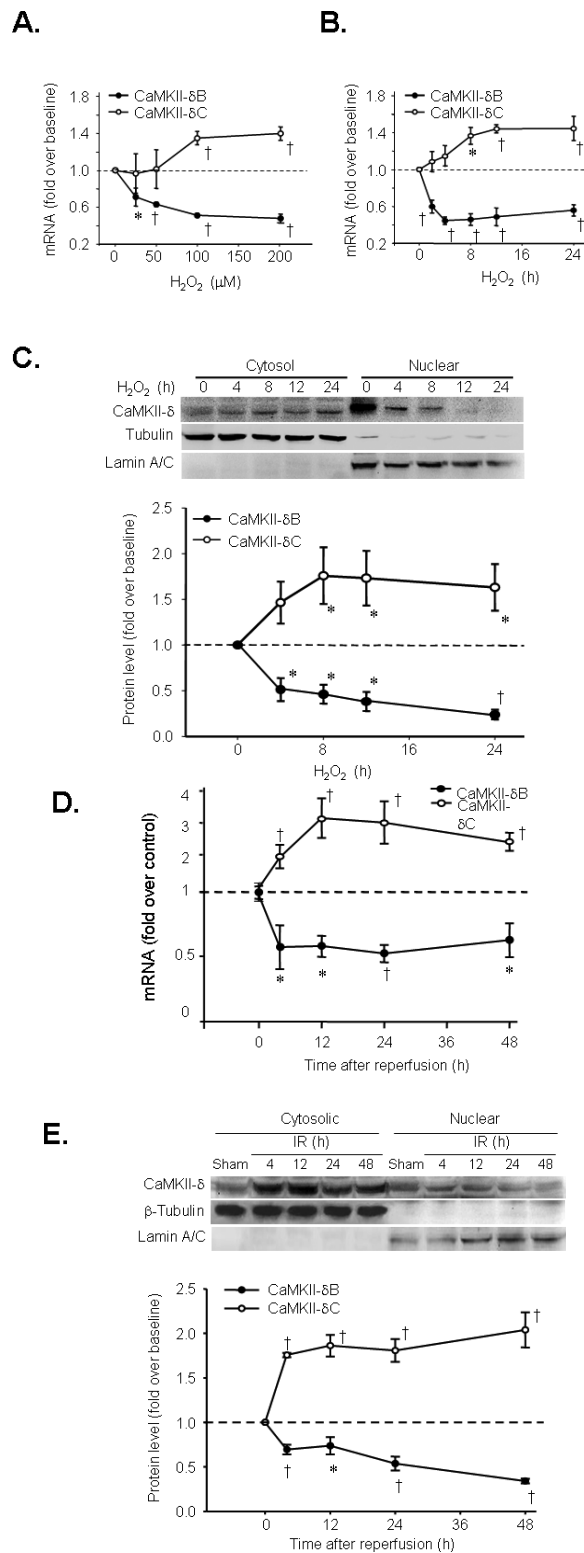


Figure 1. CaMKII-δB and CaMKII-δC expression in neonatal rat cardiomyocytes in response to oxidative stress or in rat myocardium subjected to ischemia/reperfusion (IR) injury

A. CaMKII-δB and CaMKII-δC gene expression assayed by real-time PCR in response to different concentrations of H₂O₂ treatment for 8 h in cultured neonatal cardiomyocytes (n =

6 for each data point). **B.** CaMKII- δ B and CaMKII- δ C gene expression assayed by real-time PCR in response to H₂O₂ (200 μ M) treatment for different period of time in cultured neonatal cardiomyocytes (n = 6 for each data point). **C.** Typical Western blots and the statistic data of the time course of CaMKII- δ protein abundance in nuclear and cytosolic fractions from neonatal cardiomyocytes treated with H₂O₂ (200 μ M) (n = 4 for each time point). **D.** shows CaMKII- δ B and CaMKII- δ C mRNA levels in ischemic area of rat hearts subjected to 45 min ischemia followed by different periods of reperfusion assayed by real-time PCR (n = 6 for each time point). **E.** CaMKII- δ B and CaMKII- δ C protein levels assayed by Western blotting in nuclear and cytosolic fractions from ischemic myocardium of rat hearts (n = 3-5 for each time point). For all panels, * P<0.05; † P<0.01. vs. baseline.

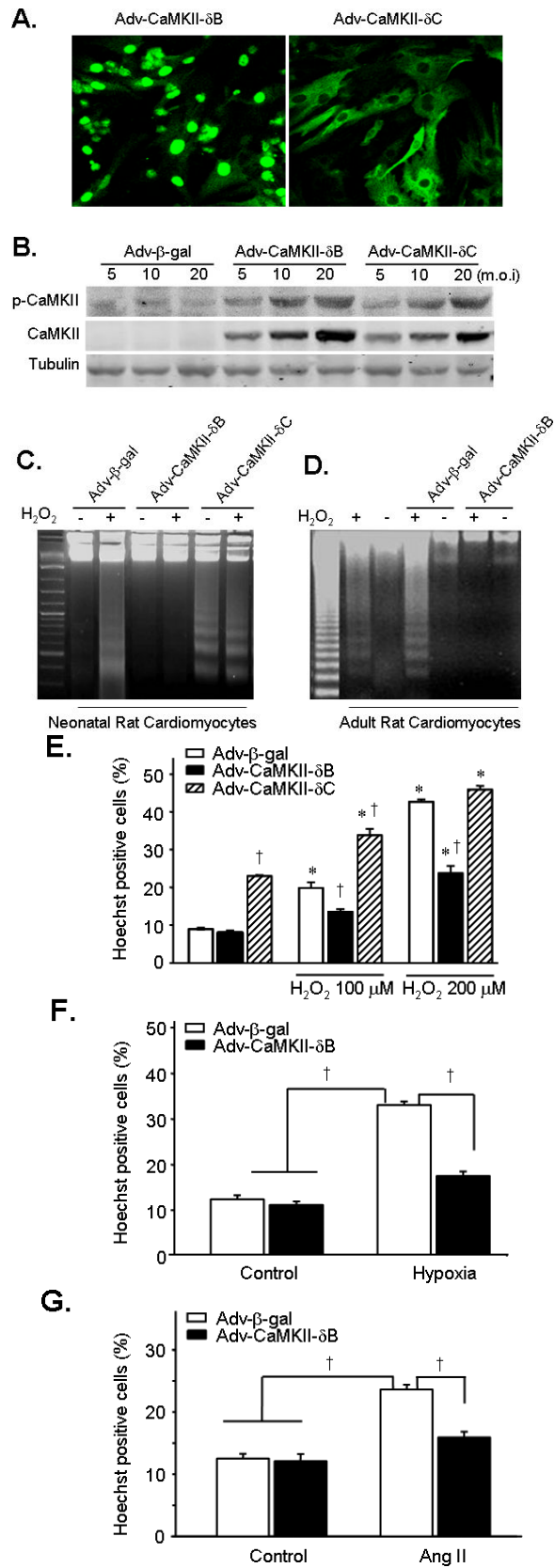


Figure 2. Overexpression of CaMKII- δ B protects both neonatal and adult rat cardiomyocytes against H₂O₂-triggered apoptosis

A. Immunofluorescent imaging with an anti-HA antibody to visualize the intracellular distribution of HA-tagged CaMKII- δ B and CaMKII- δ C in neonatal rat cardiomyocytes infected with Adv-CaMKII- δ B or Adv-CaMKII- δ C (both at 20 m.o.i. for 24 h). **B.** Typical Western blot with a site-specific antibody reacting with CaMKII-d or phosphorylated CaMKII-d in lysate from myocytes infected with Adv- β -gal, Adv-CaMKII- δ B, or Adv-CaMKII- δ C at m.o.i. as indicated for 24 h. **C.** DNA laddering in neonatal rat cardiomyocytes infected with Adv-CaMKII- δ B, Adv-CaMKII- δ C or Adv- β -gal (all at 20 m.o.i. for 24 h) then treated with H₂O₂ (200 μ M) for another 24 h. Similar results were obtained in three independent experiments. **D.** Representative DNA laddering in adult rat cardiomyocytes infected with Adv-CaMKII- δ B or Adv- β -gal (both at 100 m.o.i. for 24 h) then treated with H₂O₂ (10 μ M) for another 24 h. Similar results were obtained in three independent experiments. **E.** Average data of Hoechst staining in neonatal cardiomyocytes infected with Adv-CaMKII- δ B, Adv-CaMKII- δ C or Adv- β -gal (all at 20 m.o.i. for 24 h) then subjected to H₂O₂ (200 μ M) for 24 h. **F.** Average data of Hoechst staining in neonatal cardiomyocytes infected with Adv-CaMKII- δ B or Adv- β -gal (at 20 m.o.i. for 24 h) then subjected to hypoxia for 9 h. **G.** Average data of Hoechst staining in neonatal cardiomyocytes infected with Adv-CaMKII- δ B or Adv- β -gal (at 20 m.o.i. for 24 h) then subjected to Ang II (1 μ M for 48 h). For **E-G**, each data point shows the result from 5000-8000 cells in four independent experiments for Hoechst staining (* P<0.01 vs. Adv- β -gal in the absence of H₂O₂; † P<0.01 vs. the corresponding Adv- β -gal in each group of different concentrations of H₂O₂ or as indicated).

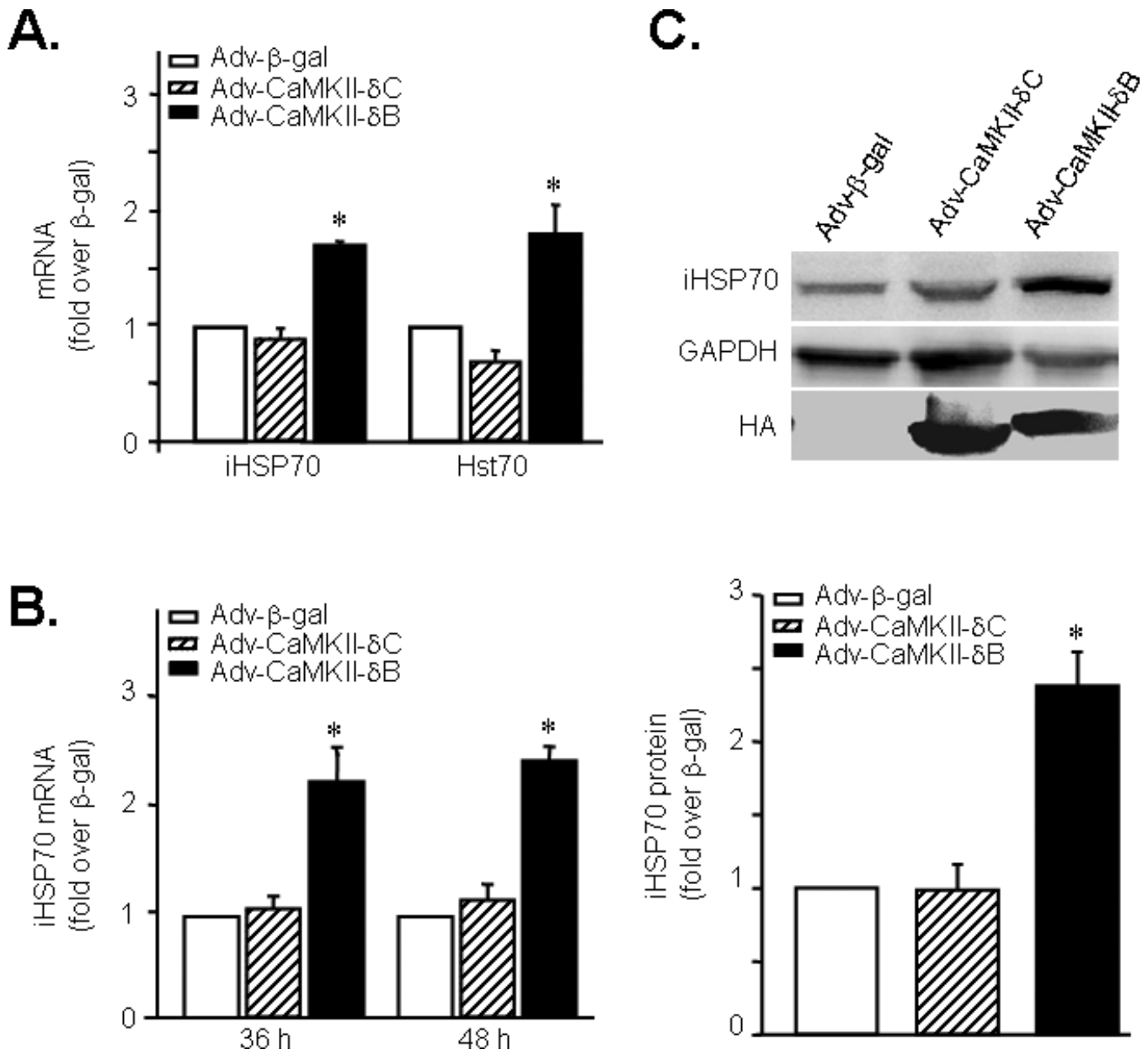


Figure 3. Upregulation of iHSP70 and Hst70 by overexpression of CaMKII- δ B but not CaMKII- δ C in cultured neonatal rat cardiomyocytes

A. iHSP70 and Hst70 gene expression assayed by cDNA microarray analysis in cells infected with Adv-CaMKII- δ B, Adv-CaMKII- δ C, or Adv- β -gal. **B.** iHSP70 gene expression assayed by real-time PCR in neonatal cardiomyocytes after adenoviral infection for 36 h or 48 h. (n = 4). **C.** Typical Western blot with an anti-iHSP70 antibody and the average data in cells infected with Adv-CaMKII- δ B, Adv-CaMKII- δ C, or Adv- β -gal (at 20 m.o.i.) for 48 h. (n = 4). For all panels, * P<0.05 vs. Adv-CaMKII- δ C and Adv- β -gal.

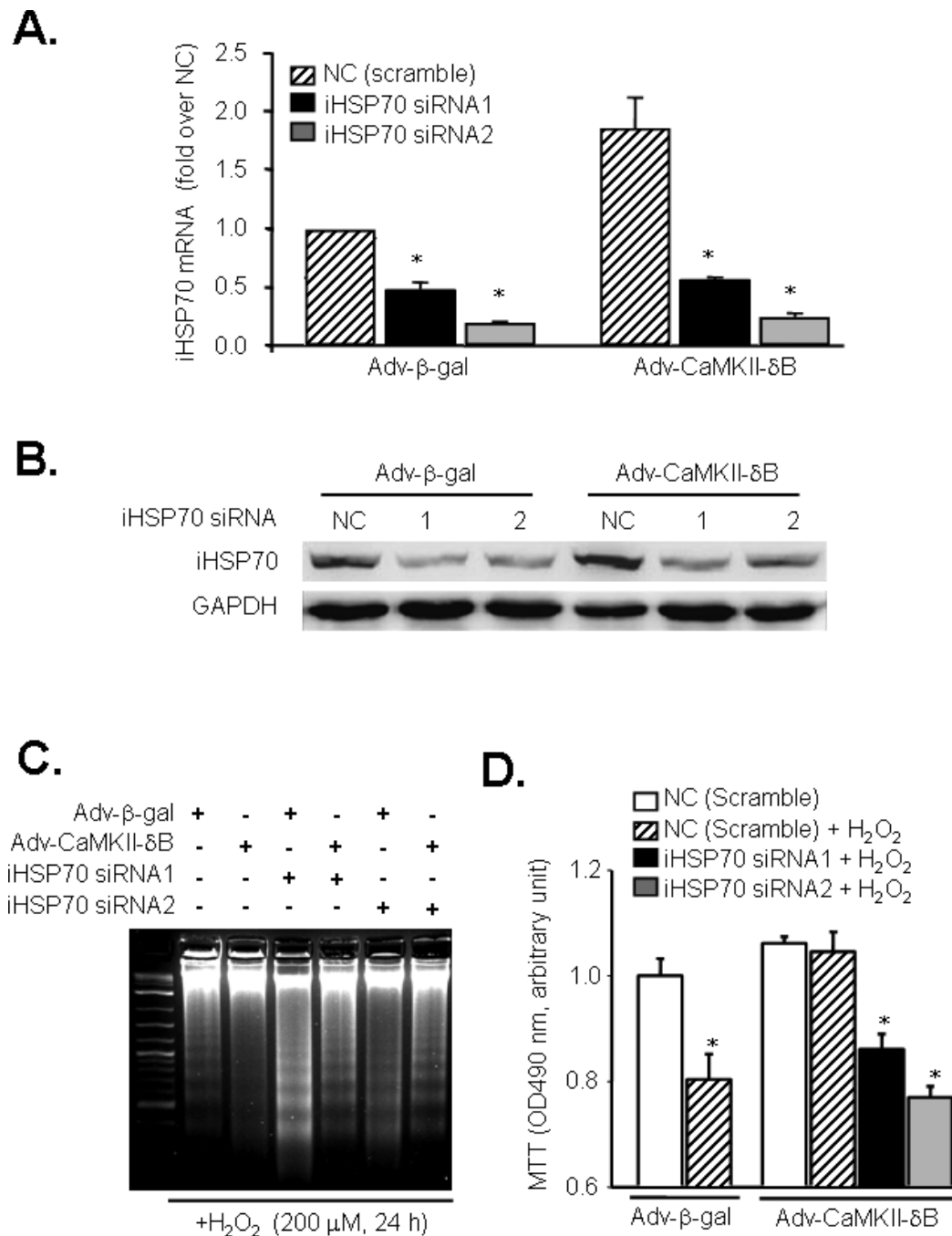


Figure 4. Gene silencing of iHSP70 blocks CaMKII-δB-mediated anti-apoptotic effect

A. siRNA-induced reduction of iHSP70 at mRNA level assayed by real-time PCR in neonatal cardiomyocytes infected with Adv-β-gal or Adv-CaMKII-δB (n = 3 for each group * P<0.05 vs. the NC of each group). **B.** siRNA-induced reduction of iHSP70 at protein level in cells infected with Adv-β-gal or Adv-CaMKII-δB. Similar results were observed in three independent experiments. **C.** DNA laddering in cells infected with Adv-β-gal or Adv-CaMKII-δB in the absence or presence of iHSP70 siRNA after H₂O₂ (200 μM for 24 h) treatment. Similar results were obtained in three independent experiments. **D.** MTT cell viability assay in cells infected with Adv-β-gal or Adv-CaMKII-δB in the absence or presence of iHSP70 siRNA after H₂O₂ (200 μM for 24 h) treatment (n = 3, * P<0.05 vs. the NC of each group).

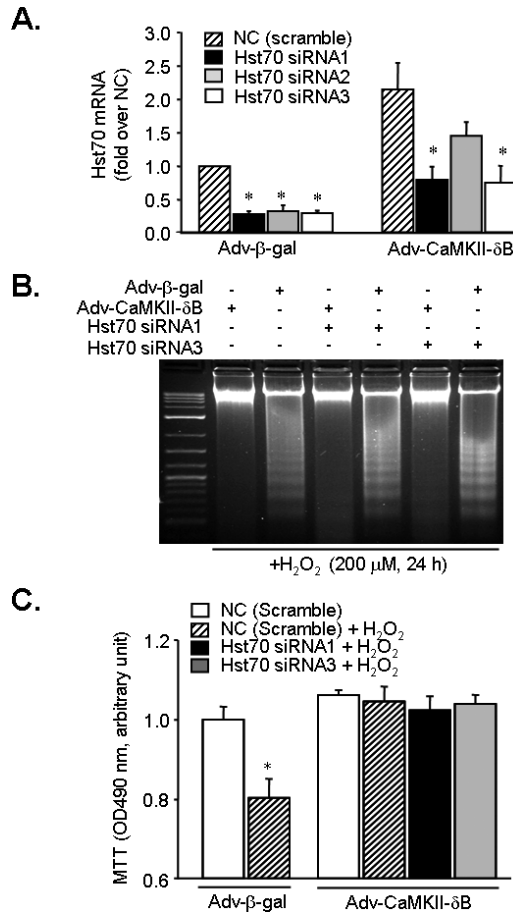


Figure 5. Gene silencing of Hst70 does not affect CaMKII-δB-mediated anti-apoptotic effect
A. siRNA-induced reduction of Hst70 at mRNA level assayed by real-time PCR in neonatal cardiomyocytes infected with Adv-β-gal or Adv-CaMKII-δB (n = 3 for each group, * P<0.05 vs. the NC of each group). **B.** DNA laddering in myocytes infected with Adv-β-gal or Adv-CaMKII-δB in the absence or presence of Hst70 siRNA after H₂O₂ (200 μM for 24 h) treatment. **C.** MTT cell viability assay in cells infected with Adv-β-gal or Adv-CaMKII-δB in the absence or presence of Hst70 siRNA after H₂O₂ (200 μM for 24 h) treatment (n = 3, * P<0.05 vs. the NC of each group).

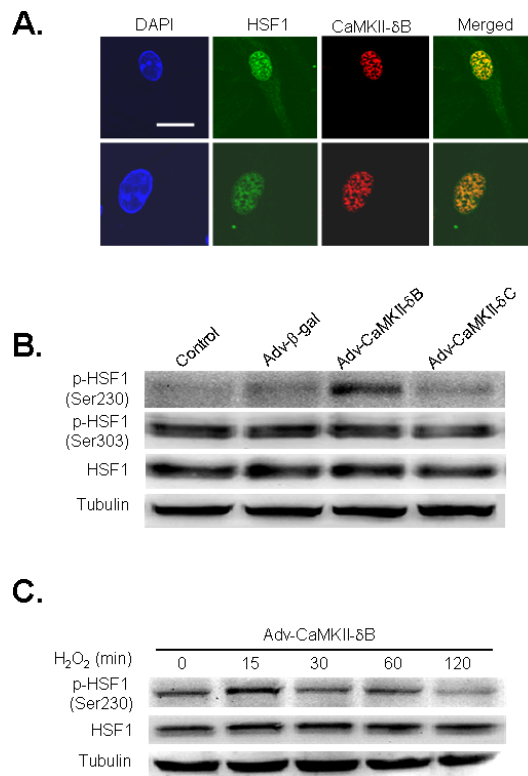


Figure 6. CaMKII-δB co-localizes with HSF1 and selectively increases phosphorylation of HSF1 at Ser230

A. Confocal immunofluorescent imaging to visualize the nucleus by DAPI staining (blue) and intracellular distribution of HSF1 (green) and HA-tagged CaMKII-δB (red) in cardiomyocytes infected with Adv-CaMKII-δB (scale bar is 20 μm). The merged imaging (yellow) shows HSF1 and HA-tagged CaMKII-δB were co-localized in the nuclear compartment. **B.** Typical western blots with an antibody reacting with total or Ser230- or Ser303-phosphorylated HSF1 in uninfected cells (Control) or those infected with Adv-CaMKII-δB, Adv-CaMKII-δC, or Adv-β-gal (at 20 m.o.i.). Similar results were obtained in other three independent experiments. **C.** Phosphorylation of HSF1 at Ser230 in neonatal cardiomyocytes infected with Adv-CaMKII-δB for 36 h and then treated with H₂O₂ (200 μM) for various time periods.

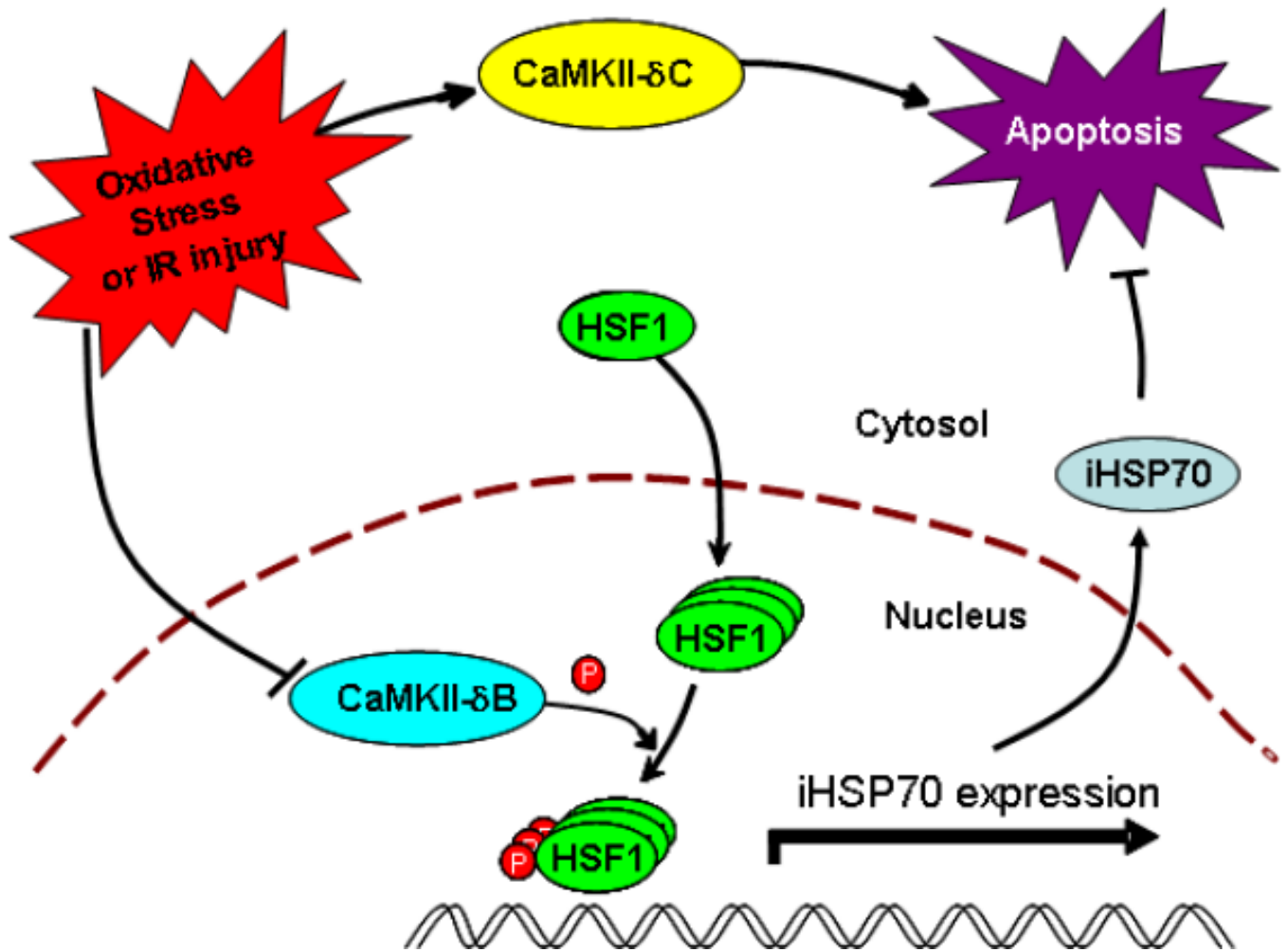


Figure 7. Schematic presentation to show oxidative stress- or IR-induced opposite regulation of the expression and functional consequence of the two major cardiac CaMKII isoforms, CaMKII-δB and CaMKII-δC

Oxidative stress or IR injury leads to a downregulation of the anti-apoptotic isoform, CaMKII-δB, and an upregulation of the apoptotic isoform, CaMKII-δC, resulting in robust cardiomyocyte apoptosis. The anti-apoptotic effect of CaMKII-δB is attributable to the kinase-mediated phosphorylation of HSF1 at Ser230 and subsequent induction of iHSP70 gene expression.