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Chronic Ca^{2+}/Calmodulin-Dependent Protein Kinase II Inhibition Rescues Advanced Heart Failure

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ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is upregulated in congestive heart failure (CHF), contributing to electrical, structural, and functional remodeling. CaMKII inhibition is known to improve CHF, but its direct cardiac effects in CHF remain unclear. We hypothesized that CaMKII inhibition improves cardiomyocyte function, $[Ca^{2+}]_i$ regulation, and β-adrenergic reserve, thus improving advanced CHF. In a 16-week study, we compared plasma neurohormonal levels and left ventricular (LV)- and myocyte-functional and calcium transient ($[Ca^{2+}]_{iT}$) responses in male Sprague-Dawley rats (10/group) with CHF induced by isoproterenol (170 mg/kg sq for 2 days). In rats with CHF, we studied the effects of the CaMKII inhibitor KN-93 or its inactive analog KN-92 ($n = 4$) (70 μg/kg per day, mini-pump) for 4 weeks. Compared with controls, isoproterenol-treated rats had severe CHF with 5-fold–increased plasma norepinephrine and about 50% decreases in ejection fraction (EF) and LV contractility [slope of LV end-systolic pressure–LV end-systolic volume relation (E_{ES})] but increased time constant of LV relaxation (τ). They also showed significantly reduced myocyte contraction [maximum rate of myocyte shortening (dL/dt_{max})], relaxation (dL/dt_{max}), and $[Ca^{2+}]_{iT}$. Isoproterenol superfusion caused significantly fewer increases in dL/dt_{max} and $[Ca^{2+}]_{iT}$. KN-93 treatment prevented plasma norepinephrine elevation, with increased basal and acute isoproterenol-stimulated increases in EF and E_{ES} and decreased τ in CHF. KN-93 treatment preserved normal myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$, and β-adrenergic reserve, whereas KN-92 treatment failed to improve LV and myocyte function, and plasma norepinephrine remained high in CHF. Thus, chronic CaMKII inhibition prevented CHF-induced activation of the sympathetic nervous system, restoring normal LV and cardiomyocyte basal and β-adrenergic–stimulated contraction, relaxation, and $[Ca^{2+}]_{iT}$, thereby playing a rescue role in advanced CHF.

SIGNIFICANCE STATEMENT

We investigated the therapeutic efficacy of late initiation of chronic Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibition on progression of advanced congestive heart failure (CHF). Chronic CaMKII inhibition prevented CHF-induced activation of the sympathetic nervous system and restored normal intrinsic cardiomyocyte basal and β-adrenergic receptor–stimulated relaxation, contraction, and $[Ca^{2+}]_i$ regulation, leading to reversal of CHF progression. These data provide new evidence that CaMKII inhibition is able and sufficient to rescue a failing heart, and thus cardiac CaMKII inhibition is a promising target for improving CHF treatment.

Introduction

Congestive heart failure (CHF) is a common, progressive, disabling, and lethal disorder. Despite improvements in treatment, the prognosis for people with CHF is still bleak (Beauverger et al., 2020; Nassal et al., 2020). Especially for those with advanced CHF, transplant remains the option of last resort, and nearly 90% die within 1 year (Sharifi-Sanjani et al., 2014; Roe et al., 2015; Gheorghiade et al., 2016; Beauverger et al., 2020; Nassal et al., 2020). Thus, the patients with the greatest need for effective therapies remain without options. The incidence and mortality rate of CHF continue to grow. New therapeutic approaches must be explored to target the pathway(s) driving CHF progression.

Disrupted cardiomyocyte Ca^{2+} homeostasis is recognized as a major contributor to the CHF phenotype. The multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) is a nodal point in regulation of intracellular Ca^{2+} handling, ion channels, and gene transcription (Anderson, 2009; Erickson et al., 2011; Singh and Anderson, 2011; Cheng et al., 2012; Roe et al., 2015). Cardiac CaMKII is upregulated in animal models and in patients with CHF. It is also associated with increased

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incidence of cardiac disease, particularly arrhythmia, progressive cardiac remodeling, and cardiac dysfunction (Hoch et al., 1999; Kirchhefer et al., 1999; Backs et al., 2009; Bers, 2010; Grimm and Brown, 2010; Sossalla et al., 2010; Singh and Anderson, 2011; Mollova et al., 2015; Hegyi et al., 2019; Nassal et al., 2020).

During the past decade, CaMKII has become a focus of studies of CHF and cardiac arrhythmia. The idea of cardiac CaMKII inhibition as a novel therapeutic principle (Pellicena and Schulman, 2014; Nassal et al., 2020) has been supported by many studies using transgenic animal models and CaMKII inhibitors ((Zhang et al., 2005aa) Backs et al., 2009; Sossalla et al., 2010; Cheng et al., 2012; Sharifi-Sanjani et al., 2014; Kreusser et al., 2016; He et al., 2019; Beauverger et al., 2020). Although previous studies revealed that mice with genetic deletion of CaMKIIδ were resistant to development of cardiac hypertrophy (Backs et al., 2009) and heart failure (Ling et al., 2009) induced by transverse aortic constriction, these studies did not examine how CaMKII inhibition might ameliorate CHF. Moreover, most of this research was conducted in animals without cardiac injury or with cardiac damage induced by transverse aortic constriction at the onset or early stage of CHF (Backs et al., 2009; Ling et al., 2009; Cheng et al., 2012; Kreusser et al., 2016; Mustroph et al., 2017; Neef et al., 2018). As a result, no clear therapeutic strategies or preclinical studies of CHF treatment have emerged, and no studies have examined late-stage chronic heart failure.

The aim of this study was to explore the impact of CaMKII inhibition in the chronic left ventricular (LV) remodeling and functional decompensation of CHF. We investigated the effects of late initiation of KN-93 (a CaMKII inhibitor) in rats with isoproterenol-induced advanced CHF, a model that mimics many structural, functional, and hormonal changes of clinical CHF (Suzuki et al., 1998; Grimm et al., 1999; Zhang et al., 2017; Li et al., 2020). We tested the hypothesis that chronic CaMKII inhibition could lead to regression of CHF by: 1) preventing CHF-induced activation of the sympathetic nervous system; 2) restoring normal LV systolic and diastolic functional performance and cardiac efficiency; and 3) improving intrinsic myocyte contraction, relaxation, calcium transient ($[Ca^{2+}]_{iT}$), and β-adrenergic reserve.

Materials and Methods

This study was approved by the Wake Forest School of Medicine Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 8th Edition, updated 2011). The experimental procedures are illustrated in Fig. 1. Briefly, 38 age-matched male Sprague-Dawley rats (Charles River Laboratories International, Inc.) were randomly divided into two groups. In the treatment group, CHF was induced by two subcutaneous injections of isoproterenol (170 mg/kg) 24 hours apart (Chen et al., 2010; Li et al., 2018, 2020). A total of 24 rats survived (~15% mortality within 48 hours); these comprised the CHF group. A second group underwent insertion of a micro-osmotic pump but no drug (controls; n = 10).

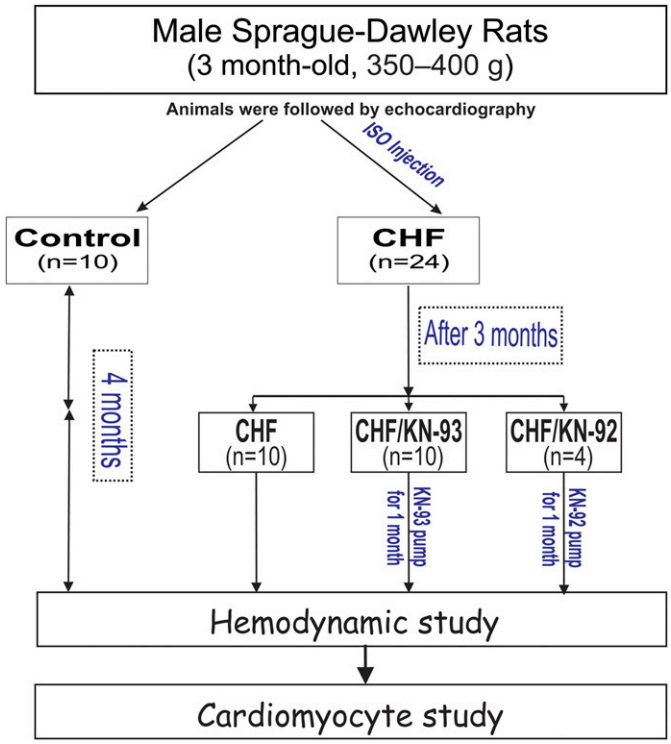

Fig. 1. A schematization of the experimental procedures and experimental timeline.

Based on previously well-characterized histology and altered LV structure and function in isoproterenol-induced CHF (Teerlink et al., 1994) and our published serial time course studies in this model, we designed a 4-month study. Cardiac function was assessed at the beginning of the study and monthly via transthoracic echocardiography (Suzuki et al., 1998; Groban et al., 2008). High doses of isoproterenol cause time- and dose-dependent structural remodeling and cardiac dysfunction that results in CHF. All isoproterenol-treated rats had CHF by 1 month and developed severe CHF by 3 months (anorexia, impaired mobility, edema, diminished appetite, and enlarged LV systolic and diastolic dimensions and concomitant reduced fractional shortening).

Four months after isoproterenol injection, rats were randomly divided into three groups: 1) no additional treatment (n = 10); 2) KN-93, a specific inhibitor of CaMKII (70 μg/kg per day square via an implanted mini-osmotic pump; Alzet, model 1004) (n = 10); and 3) KN-92 (70 μg/kg per day via mini-pump) (n = 4), an inactive KN-93 analog without CaM kinase inhibitory activity. The KN-92 group was a negative control to rule out off-target effects (Rodriguez-Mora et al., 2005; Pellicena and Schulman, 2014; Warren et al., 2017; Carrozzini et al., 2019). KN-93 doses were based on our initial concentration-response studies and past reports by us and others (Chen et al., 2010; Li et al., 2016) in which KN-93 inhibited CaMKII in mice and rats in vivo but had no effects on heart rate and end-systolic pressure. Rats were housed and fed under identical conditions and treated for

ABBREVIATIONS: β-AR, β-adrenergic receptor; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; $[Ca^{2+}]_{iT}$, calcium transient; BDNF, brain-derived neurotrophic factor; CHF, congestive heart failure; dL/dt_{max}, maximum rate of myocyte shortening; dR/dt_{max}, maximum rate of myocyte relengthening; dV/dt_{max}, peak rate of mitral flow; E_A, effective arterial elastance; E_{ES}, slope of P_{ES}-V_{ES} relation; E_{ES}/E_A, LV-arterial coupling; EF, ejection fraction; HF, pressure-overload heart failure; $I_{Ca,L}$, L-type Ca^{2+} current; ISO, isoproterenol; LV, left ventricle; τ, time constant of LV relaxation; M_{SW}, slope of stroke work-V_{ED} relation; P, LV pressure; P_{ED}, LV end-diastolic pressure; P_{ES}, LV end-systolic pressure; SA, myocyte percent shortening; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SV, stroke volume; SW, stroke work; SW/PVA, LV mechanical efficiency; TrkB, tropomyosin-related kinase receptor B; V, LV volume; V_{ED}, LV diastolic volume; V_{ES}, LV end-systolic volume.

1 month. At the conclusion of the study, rats were sacrificed, and body, heart, and lung weights were obtained.

Experimental Protocol

First, studies were performed in control rats to determine hemodynamics, LV contractility, LV diastolic filling, and LV-arterial coupling as well as mechanical efficiency, cardiac reserve (measured as response to acute β-adrenergic stimulation by isoproterenol), and neurohormonal levels. Second, to determine the cellular basis of LV functional responses to chronic KN-93 treatment, we examined freshly isolated LV myocytes from the same rats to measure cell contraction, relaxation, and $[Ca^{2+}]_{iT}$ responses and β-adrenergic reserve.

Data acquisition and analysis were not blinded except for measurements by the Hypertension Center Core Laboratory. To ensure rigor/reproducibility, we randomized age- and weight-matched rats to experimental conditions and used validated and reliable state-of-the-art techniques. In addition, echocardiograms were assessed by the same person (D.K.), and LV function was measured using pressure-volume Millar catheters (M.F.C.). For myocyte studies, we have consistently obtained reproducible, high-yield (~80%–90%), viable myocytes (maintained rod-shaped morphology for 16–18 hours). About 50–60 myocytes from each experiment were measured. Three different team members analyzed the data.

Studies in Intact Rats

Hemodynamic, LV Pressure-Volume Relationships, and LV Filling Measurements. Rats were anesthetized, intubated, and ventilated as we described previously (Shao et al., 2016; Zhang et al., 2017; Li et al., 2020). For drug infusion, a polyethylene catheter was placed into the left external jugular vein. After adequate calibration, a 2-F microtip P-V catheter (SPR-869; Millar Instruments, Houston, TX) was inserted through the right carotid artery into the LV apex using a closed-chest approach, After stabilization, signals were continuously recorded at a sampling rate of 500 samples/s using a P-V conductance system (MPCU-200; Millar Instruments) with BioBench software (National Instruments, Inc.). First, steady-state and inferior vena cava occlusion data were collected at baseline. Then acute ISO (10^{-8} M, 0.5 ml i.v.) was infused. Steady-state and vena cava occlusion data were continuously recorded immediately and during 10- to 15-minute periods. Changes between baseline and after isoproterenol were defined as cardiac reserve.

Using the instrument's software (PVAN; Millar Instruments), we measured standard steady-state hemodynamic data, such as heart rate, LV pressure (P), the time constant of LV relaxation (τ), LV volume (V), and the maximum rate of change of LV volume [peak rate of mitral flow (dV/dt_{max})]. Stroke volume (SV), cardiac output, and stroke work (SW) were calculated and corrected according to in vitro and in vivo volume calibrations. LV P-V relationships and slopes were generated. Effective arterial elastance (E_A) was calculated as the ratio of LV end-systolic pressure (P_{ES}) and SV, and LV-arterial coupling was quantitated as the ratio of the slope of P_{ES}–LV end-systolic volume relation (E_{ES}) to E_A (Segers et al., 2005; Cheng et al., 2006; Radovits et al., 2009). LV PVA was determined as the area under end-systolic P-V relation and systolic P-V trajectory above LV end-diastolic pressure (P_{ED})–LV diastolic volume (V_{ED}) curve. Efficiency of the conversion of mechanical energy to external work of the LV (i.e., cardiac mechanical efficiency) was calculated as SW/PVA (Nozawa et al., 1994; Li et al., 2020).

Plasma Catecholamine and 8-Isoprostane Levels. As previously described, blood was put into chilled tubes containing EDTA, separated on a centrifuge, and stored at −20°C. Plasma levels of norepinephrine and 8-isoprostane (to reflect systemic oxidative stress levels) were measured by the Hypertension Center Core Laboratory at Wake Forest School of Medicine (Fam and Morrow, 2003; Shao et al., 2016). All assays were performed in duplicate.

Isolated Cardiomyocyte Studies

Myocyte Isolation. After the hemodynamic study, rats were deeply anesthetized, and the hearts were excised and immediately placed in ice-cold calcium-free HEPES buffer solution. Calcium-tolerant, high-yield myocytes were obtained as we previously described (Cheng et al., 2006; Shao et al., 2016; Li et al., 2018). Cells were suspended in a modified HEPES solution ("the study buffer") with 1.2 mM $CaCl_2$ and stored at room temperature until ready for use. After 2 hours of stabilization, LV myocytes were counted, and their viability and morphology were evaluated. From each experiment, 50–60 rod-shaped cells were randomly selected for measurement of cardiomyocyte dimensions. These myocytes were used within 10–14 hours.

Myocyte Function Evaluation

Myocyte Contractile Function at Baseline and Response to Acute β-Adrenergic Receptor Agonist. After stabilization, freshly isolated cardiomyocytes were placed in superfused culture dishes. Myocyte contraction was elicited by field stimulation (0.5 Hz). A fluorescence and contractility system (IonOptix, Milton, MA) was used to measure functional performance. First, we recorded steady-state baseline data. Then data were acquired during superfusion of isoproterenol (10^{-8} M) for 8–10 minutes and after drug washout. Changes between baseline and postsuperfusion function were defined as myocyte β-adrenergic reserve. The myocyte percent shortening (SA), maximum rate of myocyte shortening (dL/dt_{max}), and maximum rate of myocyte relengthening (dR/dt_{max}) were derived as previously reported (Shao et al., 2016; Li et al., 2018).

Simultaneous Measurement of Contractile and Calcium Transient Responses. Myocytes were incubated with 10 mM indo-1-AM (Molecular Probes, Eugene, OR) and then placed in a flow-through dish. Contractile and $[Ca^{2+}]_{iT}$ responses in a single cell were measured simultaneously with a dual-excitation fluorescence photomultiplier system (IonOptix) (Morimoto et al., 2004; Shao et al., 2016). After stabilization, we recorded steady-state baseline data and then repeated the isoproterenol protocol. Because compartmentalization of the indicator in mitochondria might have occurred after myocytes were loaded with indo-1-AM, the absolute value of $[Ca^{2+}]_i$ was not used. Instead, we calculated relative changes in peak $[Ca^{2+}]_{iT}$ before and after interventions as the ratio of the emitted fluorescence (Morimoto et al., 2004; Li et al., 2018).

Drugs

KN-93 (2-[*N*(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) is a cell-permeable and potent inhibitor of CaMKII. KN-93 is the most widely used inhibitor for study of cellular and in vivo functions of CaMKII. KN-92 (2-[*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) is KN93's secondary amidic analog and differs from it only in absence of a hydroxyethyl group on the sulfonamide nitrogen (Rodriguez-Mora et al., 2005; Carrozzini et al., 2019). KN-92 does not inhibit CaMKII and has been used as a negative control in studies of antagonist activities of KN-93 (Anderson et al., 1998; (Zhang et al., 2005aa); Pellicena and Schulman, 2014; Warren et al., 2017; He et al., 2019; Nassal et al., 2020).

KN-93 inhibits L-type Ca^{2+} current ($I_{Ca,L}$) and voltage-dependent K^+ current (K_v) independent of CaM kinase inhibition. It also blocks modulation of $I_{Ca,L}$ by CaMKII and has direct effects on the channel (Li et al., 1992; Anderson et al., 1998). KN-93 and KN-92 were equipotent $I_{Ca,L}$ inhibitors at the concentration (0.5 μM) used to inhibit CaMKII in isolated heart experiments (Anderson et al., 1998). In addition, KN-93 blocks macroscopic K_v in smooth muscle cells at concentrations (0.3–3 μM) used to inhibit CaMKII (Ledoux et al., 1999). KN-92 similarly blocks the channel and is therefore useful in excluding K^+ channel effects (Anderson, et al., 1998; Pellicena and Schulman, 2014). Isoproterenol hydrochloride is a nonselective β-adrenergic

receptor (β-AR) agonist. All drugs were obtained from Tocris Bioscience (Minneapolis, MN).

Statistical Analysis

All data are presented as mean ± S.D. ANOVA was used to compare LV function, systemic hemodynamics, neurohormonal profiles, and myocyte function among the groups. When the ANOVA showed significant differences, a Bonferroni adjustment was used to compare pairwise tests among each group. Treatment effects were determined by ANOVA on the outcome measures adjusted for baseline values. Myocyte contraction, relaxation, and $[Ca^{2+}]_{iT}$ values of each rat were averaged and treated as a single data point. The mean differences in cell dynamics and the indo-1-AM fluorescence ratios between groups were calculated. Significance was set at $P < 0.05$.

Results

Verification of Experimental CHF

Isoproterenol-treated rats had CHF at 1 month, which progressed to advanced CHF by 4 months. These rats showed clear signs of clinical features of CHF, including significant increases in heart weight and the ratio of heart weight to body weight (Table 1). Compared with controls, LV ejection fraction (EF) and LV contractility (E_{ES}) were reduced by about 50%, whereas the τ, LV end-diastolic pressure, and LV end-diastolic volume were significantly increased in CHF (Fig. 3; Fig. 5A; Table 2). These LV abnormalities were accompanied by intrinsic defects of LV myocyte force-generating capacity and relaxation, as indicated by >40% decreases in myocyte peak velocity of shortening (dL/dt_{max}) and peak velocity of relengthening (dR/dt_{max}) and significantly reduced peak systolic $[Ca^{2+}]_{iT}$ (Fig. 6A; Table 3). These findings documented advanced CHF in this model.

LV Function, Cardiac Reserve, and Hormonal Activation in CHF: Effects of CaMKII Inhibition

Hormone Levels and LV Systolic and Diastolic Function at Baseline. Plasma levels of norepinephrine increased by about 5-fold and 8-isoprostane concentrations were doubled in CHF groups versus controls, consistent with significant activation of the sympathetic nervous system and increased oxidative stress (Fig. 2). As shown in Table 2, there were no differences in heart rate and P_{ES} among groups. In CHF animals, P_{ED}, V_{ES}, and V_{ED} were all significantly increased and accompanied by significantly reduced LV peak filling rate (dV/dt_{max}) and SV.

CHF groups showed a progressive decrease in basal LV contractility (Fig. 3). The slopes of LV P-V relations of E_{ES} (load-insensitive measures of LV contractile performance) were decreased by 49%. LV-arterial coupling (E_{ES}/E_A) and the efficiency of conversion of mechanical energy to external work of the heart (SW/PVA) were also significantly reduced by ~35%. In contrast, LV systolic and diastolic functional performance and general hemodynamics (Fig. 5A; Table 2) were similar among groups. KN-93 treatment prevented decreased LV contractility of E_{ES}, EF, and the abnormal upward and rightward shifts of LV P-V loops (Fig. 3C). KN-93 treatment also totally abolished the diminished cardiac mechanical efficiency of SW/PVA seen in CHF. τ, P_{ED}, V_{ED}, SV, dV/dt_{max}, E_{ES}/E_A, and cardiac weight and the ratio of heart to body weight were all close to control values (Fig. 5A; Tables 1 and 2). KN-93 treatment corrected the abnormal elevations of plasma levels of norepinephrine and 8-isoprostane caused by CHF (Fig. 2). By contrast, KN-92 treatment did not improve LV functional performance; abnormal alterations in EF, E_{ES}, τ, P_{ED}, V_{ED}, SV, LV filling, and cardiac weights; ratios of heart to body weights; or elevated plasma levels of norepinephrine and 8-isoprostane (Fig. 2; Fig. 5A; Table 1).

LV Systolic and Diastolic Functional Responses to Acute β-Adrenergic Stimulation. As shown in Table 2, acute isoproterenol infusion caused significant increases in heart rate, EF, dV/dt_{max}, and SV but significant decreases in V_{ES} and τ. After isoproterenol, P_{ES}-V_{ES} relationships were shifted to the left with significant increases in LV contractility and LV cardiac efficiency, as shown by SW/PVA (Fig. 3; Fig. 5B). By contrast, as expected, CHF rats had significantly attenuated LV positive inotropic and lusitropic responses to β-AR stimulation after acute isoproterenol infusion, demonstrating diminished cardiac β-adrenergic reserve. KN-93 in these rats completely restored the normal β-adrenergic reserve. Compared with controls, acute isoproterenol treatment resulted in similarly significant increases in heart rate, EF, dV/dt_{max}, and SV and LV contractility (measured as E_{ES} and M_{SW}) but significant decreases in V_{ES}, V_{ED} and τ. Cardiac efficiency as measured by SW/PVA was also normalized (Fig. 3; Fig. 5B; Table 2). KN-92 treatment did not improve β-AR reserve, and LV positive inotropic and lusitropic responses to β-AR stimulation during acute isoproterenol infusion remained significantly attenuated (Fig. 5B).

Myocyte Function, β-AR Reserve, and $[Ca^{2+}]_i$ Regulation in CHF: Effects of CaMKII Inhibition

LV Myocyte-Functional Performance and $[Ca^{2+}]_{iT}$ Response at Baseline. Fig. 4; Fig. 6A and Table 3 showed basal cell contractile function and $[Ca^{2+}]_{iT}$ responses in cardiomyocytes. Compared with controls, the length of myocytes (HF: 141.5 vs. Control: 115.7 μm, $P < 0.01$) and the length-width ratio were significantly increased in CHF rats. After KN-93 treatment, LV myocyte SA, dL/dt_{max}, dR/dt_{max}, and $[Ca^{2+}]_{iT}$ all recovered to control values, and myocyte length and the length-width ratio were normalized. Myocyte contractile and relaxation dysfunction, impaired $[Ca^{2+}]_{iT}$, and myocyte shape remodeling persisted after KN-92 treatment (Fig. 4; Fig. 6A). These data further confirm that the beneficial action of KN-93 is due to inhibition of CaMKII.

Myocyte-Functional and $[Ca^{2+}]_{iT}$ Responses to Acute β-Adrenergic Stimulation. Compared with controls, functional performance of myocytes in CHF rats was impaired at baseline (Fig. 6A). Furthermore, increases in myocyte contractility after isoproterenol were also significantly reduced. In CHF myocytes, isoproterenol-induced increases in

TABLE 1
Effects of chronic KN-93 on BW and HW after isoproterenol-induced CHF
Data shown as mean ± S.D.

	BW (g)	HW (mg)	HW/BW (mg/100 g)
Control (n = 10)	455 ± 15	1413 ± 53	314 ± 17
CHF (n = 10)	439 ± 11	1870 ± 73*	429 ± 21*
CHF/KN-93 (n = 10)	466 ± 15	1425 ± 92	308 ± 24
CHF/KN-92 (n = 4)	437 ± 19	1800 ± 71*	414 ± 25*

BW, body weight; HW, heart weight.
*$P < 0.05$ vs. control group.

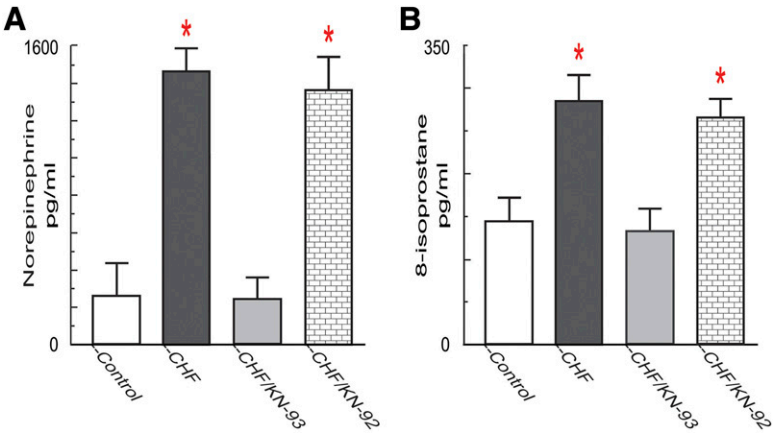

Fig. 2. Plasma levels of norepinephrine (A) and 8-isoprostane (B) in rats with and without CHF. Data are shown as mean (±S.D.). N = 6/group except for CHF/KN-92 (n = 4). *$P < 0.05$ vs. control group.

SA, dL/dt_{max}, dR/dt_{max}, and $[Ca^{2+}]_{iT}$ were all significantly lower than in control myocytes, demonstrating decreased β-adrenergic reserve (Fig. 4; Fig. 6B; Table 3). KN-93 treatment restored normal β-adrenergic reserve in CHF myocytes, but with KN-92 treatment, β-adrenergic reserve remained impaired.

Discussion

We show here, for the first time, that chronic CaMKII inhibition prevents CHF-induced activation of the sympathetic nervous system and restores normal LV systolic and diastolic function, cardiac efficiency, and β-adrenergic reserve. These are accompanied by preservation of normal intrinsic myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$, and β-adrenergic reserve. These data provide evidence that CaMKII inhibition is sufficient to rescue a failing heart, suggesting that cardiac CaMKII inhibition may provide significant benefits in CHF therapy.

Neurohormonal Activation, LV Function, and Chronic CaMKII Inhibition. Activation of the sympathetic nervous system and increased oxidative stress are general features of CHF. Increased circulating levels of norepinephrine and 8-isoprostane are seen in patients with severe CHF proportional to the degree of ventricular dysfunction and are strong inverse predictors of survival (Braunwald and Bristow, 2000). In the present study, 4 months after isoproterenol treatment, plasma levels of norepinephrine and 8-isoprostane increased by 5-fold and ~2-fold, respectively, in rats with CHF versus controls. In addition, EF and LV contractility (E_{ES}) decreased by ~50%, and cardiac mechanical efficiency shown by SW/PVA decreased by ~35%, with chronic LV remodeling and functional decompensation.

In contrast, chronic administration of the CaMKII inhibitor KN-93 restored major indices of LV systolic and diastolic functional performance and general hemodynamics (EF, τ, P_{ED}, SV, and dV/dt_{max}) to control values. To avoid the confounding effects of KN-93–induced changes in loading conditions on conventional measures of LV function, LV contractile performance was evaluated in the pressure-volume plane. KN-93 significantly increased LV contractility (measured as E_{ES} and M_{SW}) and prevented the increased τ and the abnormal upward and rightward shifts of LV P-V loops caused by CHF. LV end-diastolic volume was also significantly reduced. Chronic KN-93 treatment significantly increased the E_{ES}/E_A ratio and completely restored normal LV systolic and diastolic functional performance. This normalization may be largely attributable to prevention of excessive CaMKII activation-caused deficits in BDNF/TrkB signaling. Effective

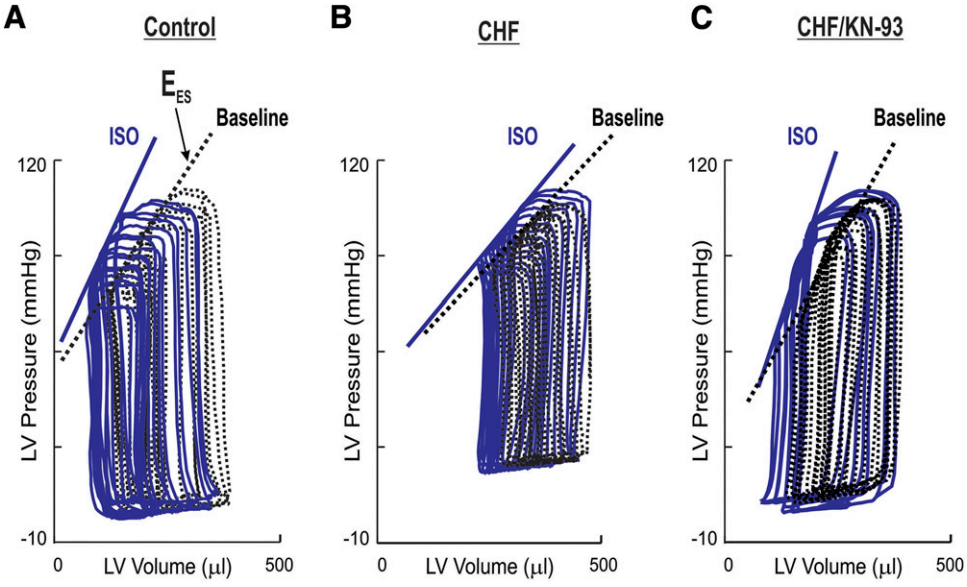

Fig. 3. Examples of P_{ES}-V_{ES} relationships at baseline (indicated by the dashed line) and isoproterenol stimulation (the solid line) in one rat from control (A), CHF (B) and CHF/KN93 (C) groups before and after isoproterenol stimulation. The P_{ES}-V_{ES} relationship is indicated by the line. The slope and position of the line provide a load-insensitive measure of LV contractility.

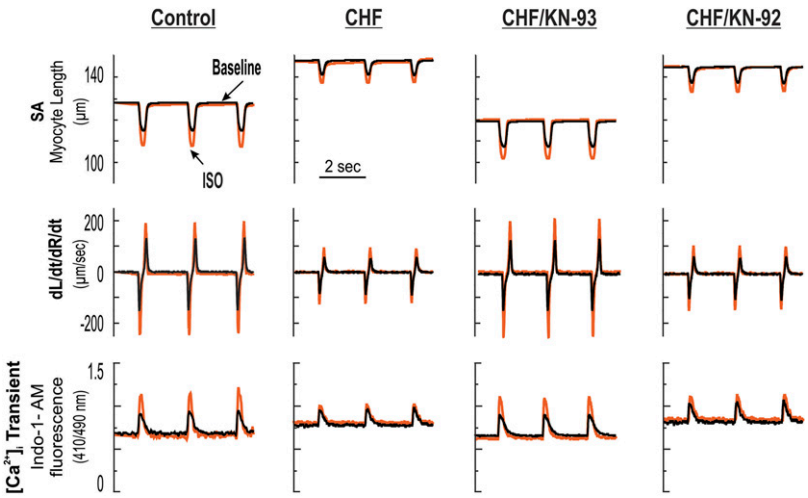

Fig. 4. Myocyte contractile function and $[Ca^{2+}]_{iT}$ response at baseline and response to acute ISO (β-adrenergic reserve). Myocytes isolated from the LV were obtained from one rat in each experimental group. Shown are superimposed traces of analog recordings of myocyte contractile and $[Ca^{2+}]_{iT}$ responses in electrically stimulated myocytes at baseline and after acute superfusion of isoproterenol (10^{-8} M).

BDNF-induced stimulation of cardiac TrkB receptors is required for cardiac contraction and relaxation, and these effects are independent of β-adrenergic stimulation. Increased activation of CaMKII is the main reason for decreased BDNF/TrkB signal transduction efficiency in CHF (Feng et al., 2015).

Chronic KN-93 also corrected abnormal elevations of norepinephrine and 8-isoprostane in CHF. These results may be attributed to improved LV function with CaMKII inhibition and likely also contributed importantly to the beneficial effects of KN-93 in CHF.

KN-93 treatment also reversed the decline of β-adrenergic cardiac reserve in CHF. Our observation is supported by previous studies using transgenic overexpression of CaMKII ((Zhang et al., 2005bb); Wagner et al., 2011). RA306, a CaMKII (δ and γ) inhibitor, reversed cardiac dysfunction in a mutant α-actin transgenic mouse model of dilated cardiomyopathy (Beauverger et al., 2020). In contrast, conditional knockout of CaMKII δ and γ induced in a low EF heart failure mouse model did not improve cardiac function but did prevent its further deterioration (Kreusser et al., 2016). In addition, CaMKIIδ knockout did not suppress severe HF induced by severe pressure overloads (Cheng et al., 2012).

Our results differ from a previous study reporting that chronic KN-93 treatment in pressure-overload heart failure (15 days after transverse aortic constriction) reversed systolic dysfunction and diminished cardiac reserve but did not improve LV diastolic function (He et al., 2019). CHF resulted in increased τ, and abnormal EDPVR persisted. By contrast, we found more pronounced improvement in LV systolic functional performance and β-adrenergic reserve and a complete reversal of CHF-caused LV diastolic dysfunction with KN-93 treatment, including normalization of LV relaxation τ, LV filling of dV/dt_{max}, and decreased P_{ED} with a downward shift of the LV P-V loop. Furthermore, LV myocyte dR/dt_{max} also returned to normal (Fig. 4; Fig. 6A; Table 3).

Several factors may contribute to these inconsistent findings. Different animal models were used (6-week-old mice vs. 12-week-old rats) with different interventions applied in early

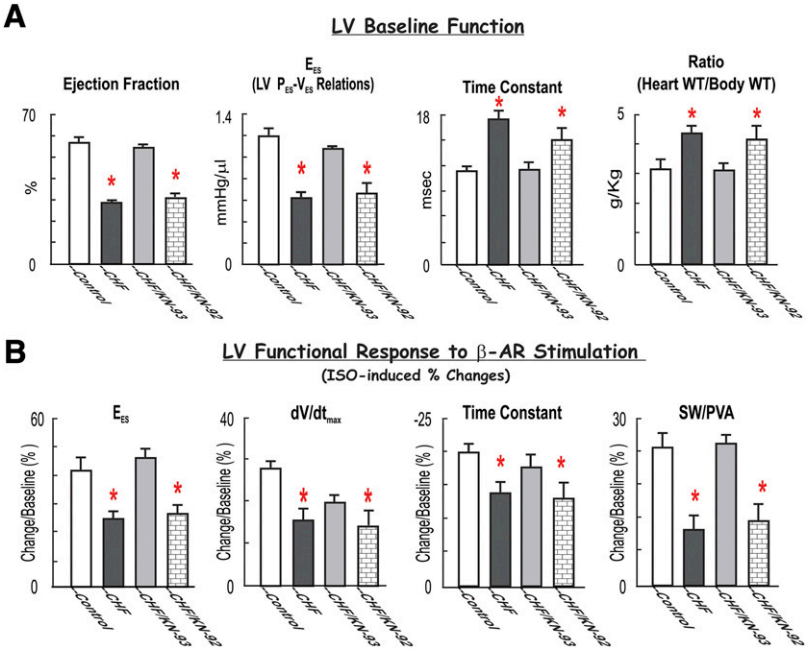

Fig. 5. (A) LV functional performance at baseline; (B) β-adrenergic reserve, calculated as % changes from baseline after acute isoproterenol stimulation. Data are shown as mean (±S.D.). $N = 6$/group except for CHF/KN-92 ($n = 4$). *$P < 0.05$ vs. control group. WT, weight.

TABLE 2
Effects of chronic KN-93 on LV function and general hemodynamic variables in CHF
Values are mean ± S.D.

	Control (n = 10)		CHF (n = 10)		CHF/KN-93 (n = 10)	
	Baseline	Isoproterenol	Baseline	Isoproterenol	Baseline	Isoproterenol
Heart rate (beats/min)	269 ± 13	307 ± 13[a]	250 ± 7	249 ± 8	255 ± 7	293 ± 4[a]
Stroke volume (μl)	174.3 ± 11.1	206.5 ± 10.7[a]	117.6 ± 4.0[b]	128.5 ± 9.4[a,c]	177.6 ± 11.0	199.0 ± 11.4[a]
LV end-diastolic pressure (mm Hg)	5.3 ± 0.3	6.3 ± 0.4	14.4.0 ± 1.1[b]	13.1 ± 0.9	4.8 ± 0.4	5.1 ± 0.2
LV end-systolic pressure (mm Hg)	110.9 ± 3.9	111.1 ± 6.0	103.6 ± 5.5	112.6 ± 5.0	112.7 ± 3.8	112.3 ± 3.7
LV end-diastolic volume (μl)	307.8 ± 13.9	314.9 ± 9.6	414.3 ± 11.2[b]	408.4 ± 17.3	339.1 ± 9.4[d]	337.2 ± 5.7
LV end-systolic volume (μl)	140.6 ± 7.6	111.2 ± 4.7[a]	296.7 ± 9.5[b]	280.0 ± 17.3[a,c]	161.5 ± 2.0[d]	138.2 ± 6.3[a,e]
Ejection fraction (%)	61.9 ± 2.6	69.5 ± 2.1[a]	31.6 ± 0.9[b]	35.4 ± 2.3[a,c]	60.2 ± 1.5	68.9 ± 2.2[a,e]
dV/dt_{max} (μl/s)	5517 ± 345	7070 ± 520[a]	3590 ± 108[b]	4171.3 ± 204.6[a,c]	5783 ± 523	6899 ± 471[a]
Time constant of relaxation (ms)	11.0 ± 0.6	8.8 ± 0.7[a]	17.8 ± 1.1[b]	15.1 ± 2.9[a,c]	11.5 ± 0.9	9.4 ± 0.3[a]
Arterial elastance (mm Hg/μl)	0.66 ± 0.05	0.56 ± 0.06[a]	0.88 ± 0.04[b]	0.78 ± 0.09[a,c]	0.64 ± 0.03	0.57 ± 0.03[a]
E_{ES} (mm Hg/μl)	1.19 ± 0.07	1.69 ± 0.09[a]	0.61 ± 0.05[b]	0.73 ± 0.06[a,c]	1.12 ± 0.02	1.66 ± 0.01[a]
M_{SW}	113.6 ± 2.8	146.8 ± 4.3[a]	65.9 ± 11.6[b]	73.2 ± 3.1[a,c]	101.9 ± 2.3	138.4 ± 2.5[a]
SW/PVA	0.71 ± 0.08	0.89 ± 0.09[a]	0.46 ± 0.06[b]	0.51 ± 0.09[a,c]	0.69 ± 0.05	0.87 ± 0.04[a]

[a] $P < 0.05$, Isoproterenol response vs. corresponding baseline value.
[b] $P < 0.05$, CHF baseline vs. control baseline.
[c] $P < 0.05$, Isoproterenol-induced percent changes between CHF vs. control.
[d] $P < 0.05$, CHF/KN-93 baseline vs. control baseline.
[e] $P < 0.05$, Isoproterenol-induced percent changes between CHF/KN-93 vs. control.

versus late stages of CHF. Furthermore, KN-93 dosing and duration also differed (once-daily injection for 1 week vs. infusion for 1 month). Nevertheless, that work and the present study both demonstrate a similar benefit of KN-93 on LV systolic function and cardiac reserve in CHF.

Myocyte Function, $[Ca^{2+}]_i$ Regulation, and Chronic CaMKII Inhibition. What is the mechanism of the restoration of normal LV systolic and diastolic function in CHF after chronic KN-93 treatment? Since the protective effects are present in freshly isolated myocytes, this beneficial action is not due to extracardiac factors, such as alterations of heart rate, fibrosis, and loading conditions, but it is directly attributable to changes in LV myocytes.

In the current study, in isoproterenol-induced CHF, LV chamber abnormalities occurred in parallel with progressive LV myocyte dysfunction, with significantly depressed myocyte contractility (dL/dt_{max}) relaxation rate (dR/dt_{max}), and $[Ca^{2+}]_{iT}$. There was a maladaptive remodeling of LV myocyte shape. These changes were normalized, and myocyte β-AR desensitization reversed after KN-93 treatment. In the KN-92 group, adverse myocyte remodeling, decreased myocyte basal and ISO-stimulated functional, and $[Ca^{2+}]_{iT}$ responses persisted. Normalization of basal and β-AR–stimulated Ca^{2+} handling may be the primary driver for reversal of CHF-caused intrinsic defects of myocyte force-generating capacity and relaxation after chronic KN-93 treatment. Recovering normal $[Ca^{2+}]_i$ regulation by chronic CaMKII inhibition may be the key mechanism for reversal of CHF-caused intrinsic defects of myocytes and rescuing advanced CHF.

In CHF, excessive activation of the sympathetic nervous system and increased oxidative stress cause upregulation of cardiac CaMKII, which contributes to abnormal myocyte Ca^{2+}

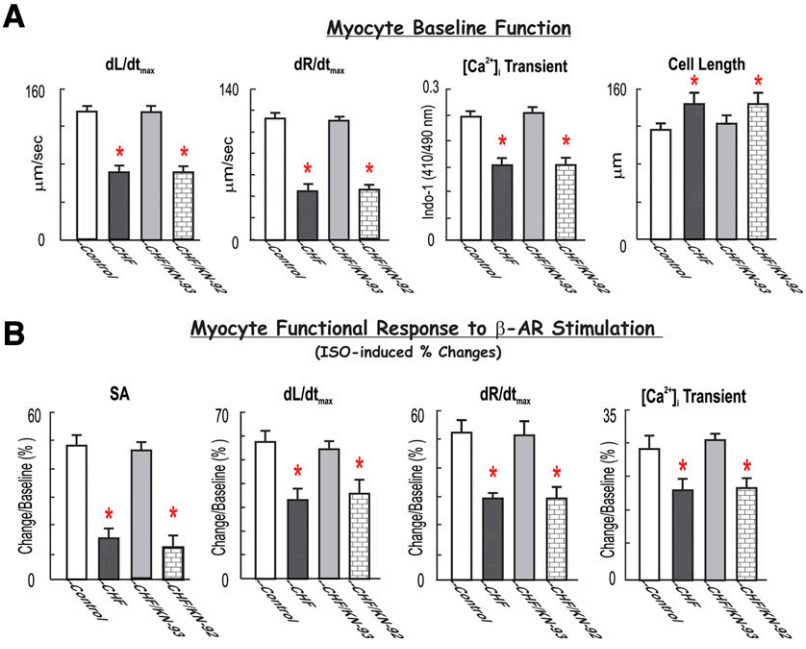

Fig. 6. (A) Functional performance of dL/dt_{max}, dR/dt_{max}, and $[Ca^{2+}]_{iT}$ at baseline; (B) β-adrenergic reserve, calculated as % changes from baseline after acute isoproterenol stimulation. Data shown as mean (±S.D.). N = 6/group except for CHF/KN-92 (n = 4). *$P < 0.05$ vs. control group.

TABLE 3
Effects of chronic KN-93 on myocyte contractile function and $[Ca^{2+}]_i$ transient in CHF
Values are mean ± S.D.

	Control (n = 10)		CHF (n = 10)		CHF/KN-93 (n = 10)	
	Baseline	Isoproterenol	Baseline	Isoproterenol	Baseline	Isoproterenol
Resting length (μm)	115.7 ± 2.5	115.1 ± 2.5	141.5 ± 0.6[a]	140.0 ± 5.0	122.1 ± 2.4	121.8 ± 5.8
Percent of shortening (SA, %)	9.6 ± 0.2	14.5 ± 0.4[b]	5.2 ± 0.6[a]	6.1 ± 0.8[b,c]	9.9 ± 0.3	14.7 ± 0.9[b]
Velocity of shortening (μm/s)	129.6 ± 5.0	206.8 ± 5.8[b]	70.9 ± 6.1[a]	96.0 ± 4.6[b,c]	133.8 ± 3.5[d]	207.8 ± 2.7[b]
Velocity of relengthening (μm/s)	110.5 ± 4.5	171.3 ± 8.5[b]	45.0 ± 4.1[a]	58.3 ± 6.3[b,c]	109.3 ± 3.0	169.7.8 ± 7.2[b]
Peak systolic $[Ca^{2+}]_i$ transient	0.24 ± 0.01	0.32 ± 0.01[b]	0.15 ± 0.01[a]	0.18 ± 0.02[b,c]	0.25 ± 0.01	0.33 ± 0.03[b]

[a] $P < 0.05$, CHF baseline vs. control baseline.
[b] $P < 0.05$, Isoproterenol response vs. corresponding baseline value.
[c] $P < 0.05$, Isoproterenol-induced percent changes between CHF vs. control.
[d] $P < 0.05$, CHF/KN-93 baseline vs. control baseline.

homeostasis by altering key proteins involved in Ca^{2+} handling, excitation-contraction coupling, cardiomyocyte function, and hypertrophy. These include the ryanodine receptor, phospholamban, L-type Ca^{2+} channel, Na^+/Ca^{2+} exchanger, and SERCA2a (Erickson et al., 2008; Anderson, 2009; Beauverger et al., 2020). We and others have reported that CHF and CamKIIδ overexpression in transgenic mice (Maier et al., 2003) decreased SERCA2a expression and activity in LV myocytes, with increased sarcolemmal Na^+-Ca^{2+} exchange expression accompanied by increased sarcoplasmic reticulum Ca^{2+} leakage and defective Ca^{2+} removal. CaMKII inhibition with KN-93 prevents CHF and cardiac injury–caused downregulation of cardiac SERCA2a and reduced $[Ca^{2+}]_{iT}$ (Cheng et al., 2019). A CaMKII inhibitor effectively reduced diastolic sarcoplasmic reticulum Ca^{2+} leak and improved Ca^{2+} transient amplitudes and contractility in ventricular myocytes from CaMKII-overexpressing mice with heart failure (Neef et al., 2018).

Previously, we have shown that in CHF and diabetic cardiomyopathy, increased activation of the sympathetic nervous system was associated with cardiac β_1-AR downregulation but β_3-AR upregulation. β_1-AR–stimulated, G_S-coupled positive inotropic effects were reduced, whereas β_3-AR–stimulated, G_i-coupled negative modulation on LV and myocyte contraction and relaxation as well as $[Ca^{2+}]_i$ regulation was enhanced. Activation of the sympathetic nervous system caused downregulation of β_1-AR, and upregulation of an inhibitory pathway mediated by β_3-ARs is responsible for β-AR desensitization in CHF (Cheng et al., 2001, 2010; Morimoto et al., 2004). Chronic KN-93 prevented diabetic cardiomyopathy-caused upregulation of CaMKIIδ and downregulation of cardiac β_1-AR and SERCA2a and reversed the increased β_3-AR and nitrotyrosine, thus preserving increased activation of the sympathetic nervous system, LV myocyte dysfunction, depressed $[Ca^{2+}]_{iT}$, and reduced β-adrenergic reserve (Cheng et al., 2019). The ability of CaMKII inhibition to counteract pathologic oxidation may be another important mechanism by which KN-93 treatment could ameliorate CHF.

Growing evidence suggests that multiple mechanisms contribute to excessive activation of CaMKII in CHF. For instance, Ca^{2+} overload and oxidative stress are general characteristics of heart failure, which activate CaMKII by Ca^{2+}/CaM-dependent and oxidation-dependent pathways (Dewenter et al., 2017). CaMKII is also a key sensor of cardiac oxidant stress. However, CaMKII activated by increased oxidant stress is different. CaMKII is activated by modification of regulatory domain methionines caused by reactive oxygen species. Oxidized CaMKII activity persists in the absence of calmodulin binding (i.e., redox signaling can uncouple CaMKII activation from Ca^{2+}/calmodulin dependence). Methionine oxidation inhibits reassociation between regulatory and catalytic subunits of CaMKII and thus enables the perpetuation of CaMKII activity.

Therefore, in the current study, significant elevations in 8-isoprostane, a key marker of oxidative stress, may produce more enhanced and sustained CaMKII activity via oxidized CaMKII (Erickson et al., 2011; Anderson, 2015). Thus, CaMKII inhibition could represent a novel form of antioxidant therapy against CHF (Luczak and Anderson, 2014). In the current study, KN-93 reversed CHF-caused changes in cardiac β_1-AR and β_3-AR expression and activity and downregulation of SERCA2a and normalized the imbalance of NCX/SERCA2a, leading to restoration of normal $[Ca^{2+}]_i$ regulation. These changes may be the key molecular mechanism for repairing LV and myocyte contractile defects, restoring cardiac β-AR responsiveness, and attenuating sympathetic overdrive. Our current observations support the view that CaMKII is a master regulator of pathways important for cardiac remodeling and dysfunction and acts as a molecular nexus linking neurohumoral stimulation to adverse cardiac remodeling and CHF.

Study Limitations. In interpreting our data, some limitations should be considered. First, we used a rat model of isoproterenol-induced CHF. Although pathologic changes in isoproterenol-treated rats are similar to those in clinical myocardial infarction (Grimm et al., 1999), and isoproterenol-induced CHF mimics many structural, functional, and neurohormonal changes of clinical CHF (Grimm et al., 1999; Carll et al., 2011), we cannot say these results are applicable to clinical CHF or other causes of CHF, such as pure pressure overload cardiomyopathy or pure volume overload cardiomyopathy. Second, we used KN-93 to study the effects of CaMKII inhibition. Although KN-93 is the most widely used inhibitor to study cellular and in vivo functions of CaMKII (Nassal et al., 2020), it inhibits $I_{Ca,L}$ and voltage-dependent K^+ (K_v), which may be independent of CaMKII actions (Anderson et al., 1998; Pellicena and Schulman, 2014). KN-92 has similar blockade effects on $I_{Ca,L}$ and K_v at the concentration used to inhibit CaMKII (Anderson et al., 1998; Ledoux et al., 1999; Warren et al., 2017). KN-92 similarly blocks the K^+ channel and is therefore useful in excluding channel-specific effects (Anderson et al., 1998; Pellicena and Schulman, 2014). Comparing the effects of KN-93 and KN-92 appears to address most known off-target effects of KN-93 (Pellicena

and Schulman, 2014; Warren et al., 2017; He et al., 2019). However, the effects of KN-93 in CHF may also be due to some currently unknown molecular effects. Third, a significant limitation of the study is that KN-93 blocks only nonphosphorylated CaMKII and appears to be an allosteric inhibitor. It does not block the catalytic activity of the enzyme, which may be a more effective approach because autonomous activity is resistant to allosteric inhibition caused by KN-93 (Vest et al., 2010). Finally, the current study did not address molecular mechanisms underlying the therapy/rescue actions of chronic CaMKII inhibition on cardiac function and cardiac reserve.

As stated in a recent review (Nassal et al., 2020), "It is reasonable to remain optimistic that targeting of CaMKII signaling has a future in cardiac translational therapy." More insights will be gained from ongoing investigations in our laboratory to elucidate these mechanisms.

Conclusions

Chronic CaMKII inhibition with KN-93 prevents CHF-induced activation of the sympathetic nervous system and restores normal LV systolic and diastolic function, cardiac efficiency, and β-adrenergic reserve. These changes are accompanied by preservation of normal intrinsic myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$, and β-adrenergic reserve. These data provide evidence that CaMKII inhibition is sufficient to rescue a failing heart and that cardiac CaMKII inhibition has promise as a therapeutic approach to combat CHF.

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Authorship Contributions

Participated in research design: Shao, H.-J. Cheng, C.-P. Cheng.

Conducted experiments: Liu, Shao, H.-J. Cheng, Li, Zhang, Callahan.

Performed data analysis: Liu, Shao, H.-J. Cheng, C.-P. Cheng.

Wrote or contributed to the writing of the manuscript: Liu, H.-J. Cheng, Callahan, Herrington, Kitzman, Zhao, C.-P. Cheng.

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