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Cardiac CaMKIIδ splice variants exhibit target signaling specificity and confer sex-selective arrhythmogenic actions in the ischemic-reperfused heart ☆

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ABSTRACT

Background: Ischemia-related arrhythmic incidence is generally lower in females (vs males), though risk is selectively increased in women with underlying cardiopathology. Ca^{2+}/calmodulin dependent kinase II (CaMKII) has been implicated in ischemia/reperfusion arrhythmias, yet the role of CaMKII in the ischemic female heart has not been determined. The aim of this study was to define the role and molecular mechanism of CaMKII activation in reperfusion arrhythmias in male/female hearts.

Methods and results: Male and female rat hearts and cardiomyocytes were subjected to multiple arrhythmogenic challenges. An increased capacity to upregulate autophosphorylated CaMKII (P-CaMKII) in Ca^{2+}-challenged female hearts was associated with an enhanced ability to maintain diastolic function. In ischemia/reperfusion, female hearts (vs male) exhibited less arrhythmias (59 ± 18 vs 548 ± 9, s, $p < 0.05$), yet had augmented P-CaMKII (2.69 ± 0.30 vs 1.50 ± 0.14, rel. units, $p < 0.05$) and downstream phosphorylation of phospholamban (1.71 ± 0.42 vs 0.90 ± 0.10, $p < 0.05$). In contrast, hypertrophic female hearts had more reperfusion arrhythmias and lower phospholamban phosphorylation. Isolated myocyte experiments (fura-2) confirmed Ca^{2+}-handling arrhythmogenic involvement. Molecular analysis showed target specificity of CaMKII was determined by post-translational modification, with $CaMKII\delta_B$ and $CaMKII\delta_C$ splice variants selectively co-localized with autophosphorylation and oxidative modifications of CaMKII respectively.

Conclusions: This study provides new mechanistic evidence that CaMKIIδ splice variants are selectively susceptible to autophosphorylation/oxidation, and that augmented generation of P-$CaMKII\delta_B$(Thr287) is associated with arrhythmia suppression in the female heart. Collectively these findings indicate that therapeutic approaches based on selective CaMKII splice form targeting may have potential benefit, and that sex-selective CaMKII intervention strategies may be valid.

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1. Introduction

Ischemic heart disease is the leading cause of death in women and men, albeit with nuanced etiology and outcomes. Ischemia-related arrhythmic incidence is generally lower in females (vs males) [1–3], although mortality is increased after an ischemic event [4]. The risk of sudden cardiac death in women (not men) substantially increases when there is a myocardial oxidative stress state, such as occurs in hypertrophic pathologies [5–7].

Experimentally, we and others have shown that non-diseased female hearts exhibit a relative resistance to acute ischemia/reperfusion pathologies [8–10], including reduction in the severity of reperfusion arrhythmias, though the mechanisms responsible have not been elucidated. This female resilience is absent when hearts have an underlying hypertrophic pathology [9] and may be related to a down-regulation of the phosphoinositide 3-kinase/Akt (PI3-K/Akt) pathway [9]. Furthermore, we and others have reported fundamental sex steroid dependent differences between males/females in excitation–contraction coupling and cardiomyocyte Ca^{2+} handling processes [11,12], with Ca^{2+} challenged female cardiomyocytes exhibiting lower operational Ca^{2+} levels [13–15].

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Given that Ca^{2+} overload is a key instigator of ischemia/reperfusion arrhythmias, interest has focused on the actions of Ca^{2+}/calmodulin dependent kinase II (CaMKII) and the therapeutic potential of its inhibition in a number of myocardial disease contexts. CaMKII is a ubiquitously expressed key signaling intermediate activated in response to alterations in cellular Ca^{2+} levels [16]. In the myocardium, CaMKII is expressed primarily as two splice variants (δ_B and δ_C) [17], and Ca^{2+}-dependent activation can be maintained through post-translational modification, including autophosphorylation and oxidation (P-CaMKII(Thr287) & ox-CaMKII(Met281/2) respectively) [18]. When active, CaMKII phosphorylates and functionally modulates many of the ion channels and transporters centrally involved in cardiac excitation-contraction coupling [16]. CaMKII activation is known to both augment loading and promote leakage of the cardiomyocyte internal sarcoplasmic reticulum (SR) Ca^{2+} store in a context specific manner [12,19–23], though selective transporter targeting processes are not yet delineated.

It is notable that studies of the role of CaMKII in arrhythmogenesis have exclusively been performed in male animal models despite the documented significant sex/gender differences in cardiomyocyte Ca^{2+} handling and ischemia/reperfusion responses. There is a surprising knowledge deficit regarding the role of CaMKII in the ischemic female heart. The lower Ca^{2+} operational levels observed in female myocytes could suggest a differential pattern of CaMKII activation associated with altered susceptibility to Ca^{2+}-dependent arrhythmogenesis.

New strategies are required to achieve sex-selective therapeutic efficacy, and CaMKII represents a high-potential interventional target. The aim of this study was to define how different CaMKII post-translational modifications mediate arrhythmias and inotropic status in response to high Ca^{2+} and ischemic challenges in male and female hearts. We demonstrate that CaMKIIδ splice variants are selectively modified by autophosphorylation and oxidation. We report that upregulation of the δ_B-associated P-CaMKII(Thr287) occurs concomitantly with a suppression of reperfusion arrhythmias in the female heart. In settings of both Ca^{2+} and ischemia provocations, we provide novel evidence that selective CaMKII post-translational modifications are associated with differential downstream signaling outcomes — and that CaMKII activation is hence not invariably associated with arrhythmogenesis, as has been previously reported to be the case (in studies which have involved male hearts only) [24]. These findings significantly refine the current view of CaMKII signaling processes. The results presented indicate that therapeutic approaches based on selective CaMKII splice form targeting may have potential, and that sex-selective CaMKII intervention strategies may be valid.

2. Methods

2.1. Animals

Male and female Sprague–Dawley (SpD) rats were obtained from the Animal Resources Centre (WA, Aus). The colony of Normal Heart Rats (NHR) and Hypertrophic Heart Rats (HHR) was derived as previously reported (see Supplementary material) [9,25,26]. All rats were age-matched (12–16 weeks) and maintained under identical conditions at the Biological Research Facility at the University of Melbourne, Australia. Experiments were conducted and animals handled in the manner specified by the NHMRC/CSIRO/ACC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997) and the EU Directive 2010/63/EU for animal experiments, with approval and oversight of the project by the University of Melbourne Animal Ethics Committee. Vaginal cytology was evaluated for estrous state and confirmed the non-cycling estrous status of female rodents (as previously described for segregated male/female housing conditions [27]). Rats were anesthetized with sodium pentobarbitone (60 mg/kg) and injected with sodium heparin (200 IU) via the femoral artery, prior to heart excision.

2.2. Cardiomyocyte Ca^{2+} transient measurements

Cardiomyocytes were isolated from hearts (SpD, NHR, HHR) by enzymatic digestion as described previously (see Supplement material) [28]. Cardiomyocytes were loaded with the Ca^{2+} fluorescent dye, Fura-2 AM (2.5 μmol/L), and superfused with a HEPES–Krebs buffer on an inverted light microscope. Cells were field stimulated and microfluorimetric measurements of cardiomyocyte Ca^{2+} were performed (IonOptix, Milton, MA, USA). Ca^{2+} transient amplitude was determined by the difference between peak systolic and diastolic Ca^{2+} ($F_{360/380}$). The rate of cytosolic Ca^{2+} removal in diastole was measured by determining the rate constant of Ca^{2+} signal decay (Tau, ms; exponential fit from 50% decay to 1/e). Male and female cardiomyocytes were subjected to one of three protocols; (i) superfused with serially increasing concentrations of $CaCl_2$ (1, 2, 3, 4, 5 mmol/L) for 3 min each (25 °C, 0.5 Hz; n = 15–18 cells, N = 6 hearts), (ii) superfused with simulated ischemia solution (136 mmol/L NaCl, 8 mmol/L KCl, 0.35 mmol/L $NaH_2PO_4 \cdot H_2O$, 1.05 mmol/L $MgSO_4 \cdot 7H_2O$, 2.0 mmol/L $CaCl_2$, 10 mmol/L HEPES, 0 mmol/L glucose, 10 mmol/L lactate, pH 6.8, N_2 gas saturation; n = 9–12 cells, N = 5–7 hearts, [29,30]) in the absence/presence of CaMKII inhibitor (KN93 hydrochloride, 2.5 μmol/L; Sigma-Aldrich, NSW, Australia), and (iii) superfused with isoproterenol (10 nmol/L) for 5 min (37 °C, 4 Hz) prior to 30 s non-paced to determine vulnerability to spontaneous contraction (n = 21–25 cells, N = 4–5 hearts). All data were analyzed off-line using IonWizard (IonOptix, Milton, MA, USA). Control experiments for the KN93 compound showed Ca^{2+} transients were not modified by the analogue agent KN92 (Fig. S1).

2.3. Isolated heart preparation

Isolated hearts were perfused aerobically and heart function was monitored and analyzed (ADInstruments, Bella Vista, NSW, Australia) as previously described (see Supplement material) [31,32] throughout one of four pathological perfusion protocols; (i) high Ca^{2+} (4 mmol/L) perfusate for 2 min, (ii) 20 min global ischemia (37.0 °C) and 2 min reperfusion, (iii) 20 min global ischemia and 10 min reperfusion in the absence/presence of the CaMKII inhibitor, KN93 hydrochloride (0.5 μmol/L; Sigma-Aldrich, NSW, Australia) for 10 min immediately prior to ischemia and throughout reperfusion (n = 8 hearts), (iv) hydrogen peroxide (200 μmol/L) for 2 min. Left ventricular pressure measurements were performed using a fluid-filled balloon connected to a pressure transducer (MLT844) and recorded on a MacLab data acquisition system (ADInstruments, Bella Vista, NSW, Australia). The balloon was inflated to produce an end-diastolic pressure of 4 mm Hg and the volume kept constant throughout the perfusion protocol. Arrhythmia quantification relied on mechanical analysis, in order to directly evaluate ventricular functional outcomes of electrical instability (rather than infer function indirectly from electrical record, [32]).

2.4. Immunoblotting

Left ventricular tissue was homogenized and fractionated (Fig. S2) as previously described [33]. Homogenate was reconstituted into 2× SDS sample buffer and equal volumes were loaded onto polyacrylamide gels for SDS-PAGE and subsequent immunoblot analysis. Antibody selection, previous validation details, and immuno-imaging methods are provided in the Supplementary material.

2.5. Statistical analysis

Results are presented as mean ± SEM. Comparisons between two groups with normally distributed data was performed with a Student's unpaired t-test. Data from experiments with two groups assessed at multiple time-points were evaluated by a one-way analysis of variance (ANOVA) with repeated measures. Experiments incorporating two groups with two characteristics were assessed by two-way ANOVA with Fisher's least significant difference (LSD) post-hoc analysis. The statistical significance of the difference between correlation coefficients was evaluated by comparing z scores to calculate the observed value of z_{obs}. Differences were considered significant at $P < 0.05$. All statistical calculations were performed using SPSS v.21.0 (SPSS, Chicago, IL).

2.6. On-line Supplementary material

Supplementary materials (Figs. S1–S8) may be located in the on-line supplement.

3. Results

3.1. Accentuated CaMKII response associated with preserved diastolic function in Ca^{2+}-challenged female myocytes and hearts

Functional evidence shows that female cardiomyocytes operate at a lower operational Ca^{2+} level than males [12,22], but the contribution of CaMKII signaling to these differences is unknown. Though the absolute concentration of cardiomyocyte CaMKII in males and females is not known, the relative basal expression of CaMKII (δ_B and δ_C analyzed either individually or combined) was comparable in male and female adult rat hearts under normoxic conditions (Figs. 1 & S3). Similarly, expression of the sarcoplasmic reticulum Ca^{2+} release channel (RyR2), sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a), phospholamban (PLB), and the SERCA2a:PLB ratio were not different in male and female hearts (Fig. 1), as were levels of ox-CaMKII(Met281/2) and

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