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Sorcin ablation plus β-adrenergic stimulation generate an arrhythmogenic substrate in mouse ventricular myocytes

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ABSTRACT

Sorcin, a penta-EF hand Ca^{2+}-binding protein expressed in cardiomyocytes, is known to interact with ryanodine receptors and other Ca^{2+} regulatory proteins. To investigate sorcin's influence on cardiac excitation-contraction coupling and its role in the development of cardiac malfunctions, we generated a sorcin knockout (KO) mouse model. Sorcin KO mice presented ventricular arrhythmia and sudden death when challenged by acute stress induced by isoproterenol plus caffeine. Chronic stress, which was induced by transverse aortic constriction, significantly decreased the survival rate of sorcin KO mice. Under isoproterenol stimulation, spontaneous Ca^{2+} release events were frequently observed in sorcin KO cardiomyocytes. Sorcin KO hearts of adult, but not young mice developed overexpression of L-type Ca^{2+} channel and Na^{+}-Ca^{2+} exchanger, which enhanced I_{Ca} and I_{NCX}. Consequently, spontaneous Ca^{2+} release events in sorcin KO cardiomyocytes were more likely to induce arrhythmogenic delayed afterdepolarizations. Our study demonstrates sorcin deficiency may trigger cardiac ventricular arrhythmias due to Ca^{2+} disturbances, and evidences the critical role of sorcin in maintaining Ca^{2+} homeostasis, especially during the adrenergic response of the heart.

1. Introduction

Sorcin (soluble resistance-related calcium-binding protein) is a 21.6 kDa protein that belongs to the penta-EF hand protein family [1,2]. The protein acquires its name because it was first found to be overexpressed in multidrug-resistant cells [3–6]. However, further studies find that sorcin has a broad expression in cardiomyocytes, neurons, pancreatic β-cells, and vascular smooth muscle [7–10], clearly transcending its potential role in multi-drug resistance. The binding of Ca^{2+} to sorcin ($K_{d,Ca}$ ~1 μM) moves EF hand 1 and 3, leading to a ~33% increase of solvent-accessible surface areas. The exposure of hydrophobic residues enables sorcin to translocate from cytosol to cellular membranes and to reach its target proteins [1,2,9,11,12]. Immunostaining shows that in the presence of Ca^{2+}, sorcin localizes at z-lines close to the T-tubules of adult mouse cardiomyocytes, which enables it to regulate proteins involved in excitation-contraction coupling (e-c coupling) [12,13]. One key target protein of sorcin is ryanodine receptor 2 (RyR2), the sarcoplasmic reticulum (SR) Ca^{2+} release channel responsible for providing most of the Ca^{2+} necessary for cardiac contraction [7]. In single channel recordings of RyR2, sorcin decreases RyR2 activity in a fast (onset of effect is less than ~15 ms) [12], reversible, and dose-dependent manner [14]. Sorcin can also stimulate SR/ER Ca^{2+} ATPase (SERCA) activity by increasing its sensitivity to Ca^{2+} and enhancing the V_{max} of its Ca^{2+} pumping rate [15]. and significantly increases the sarcolemmal Na^{+}-Ca^{2+} exchanger (NCX) in rabbit cardiomyocytes [16,17]. Lastly, sorcin's effect on the L-type Ca^{2+} channel (LTCC) is more complex: while Meyers et al. find that sorcin accelerates Ca^{2+}-dependent inactivation of I_{Ca} leading to decreased I_{Ca} integral, Fowler et al. report that sorcin stimulates voltage-dependent inactivation but slows Ca^{2+}-dependent inactivation

Abbreviation: AP, action potential; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BVT, bidirectional ventricular tachycardia; CPVT, catecholaminergic polymorphic ventricular tachycardia; DAD, delayed afterdepolarization; EAD, early afterdepolarization; e-c coupling, excitation-contraction coupling; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FDHM, full duration at half maximum; LTCC, L-type Ca^{2+} channel; NCX, Na^{+}-Ca^{2+} exchanger; PVC, premature ventricular contraction; RyR2, ryanodine receptor type 2; SERCA, SR/ER Ca^{2+} ATPase; Sorcin KO, sorcin knockout; SR, sarcoplasmic reticulum; TAC, transverse aortic constriction; VT, ventricular tachycardia

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[13,18].

The above in vitro experiments, most of which are conducted using purified exogenous sorcin protein, illustrate sorcin's effects on its target proteins but fail to profile the integral role of sorcin on e-c coupling in intact cardiomyocytes and whole hearts. As sorcin not only regulates RyR2 channels, but also influences Ca^{2+} entry and SR Ca^{2+} load by interacting with LTCC, SERCA and NCX, it is difficult to predict sorcin's global effects in intact systems. Previous research attempted to answer this question by using sorcin-overexpressing animals or cardiomyocytes, however, this approach may not be optimal because the method, time, and concentration used to expose sorcin to target proteins are critical determinants of its function. Also, a high concentration of sorcin or its long-term exposure is shown to be detrimental for cell function [13,16,19,20]. Thus, an alternative model is desired to provide novel critical insight into the role of endogenous sorcin in e-c coupling and heart function.

We generated a sorcin knockout (sorcin KO) mouse by ablating the exon 3 of *Sri*, the only sorcin-encoding gene. The sorcin KO mouse shows impaired insulin release due to ER stress in pancreatic β-cells [8], but the effects of sorcin ablation in cardiac cells have not been studied. The sorcin KO mice enabled us to study the role of sorcin on e-c coupling in intact cardiomyocytes, and to model the sorcin loss-of-function mutations found in patients/models with heart diseases [11]. By subjecting the sorcin KO mice to acute and chronic stress, we could determine the consequences of sorcin ablation and dysfunction under periods of Ca^{2+} overload. We found that sorcin KO mice were born without apparent phenotype and propagated at the expected Mendelian ratios, but displayed significantly higher incidence of cardiac arrhythmias and sudden death when subjected to stress. Hearts of adult sorcin KO mice (6-month-old), but not young mice (1-month-old) developed NCX and LTCC overexpression, which enhanced I_{NCX} and I_{Ca} density. Further, in isolated sorcin KO cardiomyocytes, isoproterenol stimulation triggered high incidence of delayed afterdepolarizations (DADs) accompanied with synchronized Ca^{2+} waves. We report for the first time that sorcin ablation plus β-adrenergic stimulation generate an arrhythmogenic substrate in ventricular myocytes that may trigger lethal arrhythmias due to global Ca^{2+} disturbances.

2. Materials and methods

2.1. Generation of sorcin KO mice

Portions of the murine *Sri* were obtained by screening the 129SV CITB BAC library (Invitrogen Inc.). A DNA fragment of *Sri* exon 1, 2, 3, 4 was cloned into pBluescriptSK (Stratagene Inc.) that contained the MC1-HSV-TK cassette. To remove axon 3, which presents in both sorcin isoforms (accession no. NM_001080974.2 and NM_025618.3), a mini targeting vector containing a loxP-flanked exon 3 and a loxP-flanked phosphoglycerol kinase (PGK) promoter-driven NEO-pGHpA cassette was generated. The mini targeting vector was transformed into the recombination-competent DY380 bacteria cells that previously transformed pBluescript-*Sri*-MC1-HSV-TK. Recombinants integrated the loxP-flanked exon 3 and loxP-flanked pGK promoter-NEO-pGHpA cassettes were selected as targeting vectors. The homologous integration of the mini targeting cassette into pBluescript-*Sri*-MC1-HSV-TK was confirmed by DNA sequencing.

The targeting vector was electroporated into mouse 129S1/SvImJ ES cells. ES cells integrated the targeting vector were selected by growth on G18. Neo^r, $GANC^r$ clones were picked, expanded, and genomic DNA were isolated from each clone. Correctly targeted colonies, 1C4, 1D12, 1E1, 2B7, 2F5, 2H11, were identified by the appearance of a 2.6 k–bp band by using the ^{32}P autoradiogram 5′ probe. One euploid clone, 1D12, was microinjected into the blastocyst to produce a chimeric founder. The male chimera was mated with 129S1/SvImJ female (F_0). The $FloxNeo^{-/+}$ mouse in F_1 generation was selected to mate with EIIa-Cre transgenic mice to excise Neo cassette as well as *Sri* exon 3 (F_2). Three primers: sorIn3Rev 5′-GAA GGC TGG CAT GGA GTG AAA GCA-3′, sorIn2For 5′-CTG ACC TCA GTC AAC CAG TAA GTA GG-3′, and Neo 5′-CGT TGG CTA CCC GTG ATA TT-3′ were used to determine genotyping of mice. The excision of *Sri* exon 3 as well as Neo cassette was confirmed by a 453-bp band in PCR, which included a 3′ loxP site, a remnant 168-bp insertion from the targeting vector and neighboring intron, while WT DNA containing exon 3 presented a 1070-bp band. The $Sri^{+/-}$ mice was backcrossed to 129S1/SvImJ mice for 10 generations to eliminate Cre recombinase. Finally two $Sri^{+/-}$ mice were crossed to get the $Sri^{-/-}$ mice.

All animal experiments were approved by the University of Michigan Institutional Animal Care and Use Committee.

2.2. Western blot

Expression of RyR2, LTCC, NCX and SERCA were measured in 6-month and 1-month-old sorcin KO mice as described before [21]. Heart frozen in liquid nitrogen was pulverized by pestle, and homogenized in lysis buffer containing 0.9% NaCl, 10 mM Tris-HCl pH 6.8, 20 mM NaF, 2 μM leupeptin, 100 μM phenylmethylsulphonyl fluoride, 500 μM benzamidine, 100 nM aprotinin at 4 °C. The sample was centrifuged at 1000 × *g* for 10 min, and the supernatant was collected. The protein concentration was measured by Bradford method. For Western blots, 50 μg of lysate suspended in Laemmlii buffer was separated by SDS-PAGE in 4–20% TGX or AnyKD precast gels (Bio-Rad). Proteins were transferred to PVDF membranes at 25 V for 16-18 h at 4 °C. Then, membranes were blocked in PBS-T containing (mM) 3 KH_2PO_4, 10 Na_2HPO_4, 150 NaCl, pH 7.2–7.4, 0.1% Tween 20) plus 5% dried skim milk. Proteins were probed with the following primary antibodies: sorcin (1:2000 custom), SERCA (1:1000, ab2861, Abcam), NCX (1:200, ab6495, Abcam), Cav1.2 (1:200, ACC-003, Alomone), RyR2 (1:2000, MA3-925, ThermoFisher). After the membrane being washed three times by PBS-T, membranes were incubated with goat anti-mouse-HRP (1:1000, 31,437, Thermo) or goat anti-rabbit-HRP (1:2000, 31,463, Thermo). Protein-antibody reactions were detected by using Super-Signal Femto ECL reagent (Thermo), and imaged by the ChemiDoc MP apparatus (Bio-Rad). Band intensity was analyzed by the ImageLab software (Bio-Rad).

2.3. Confocal Ca^{2+} imaging

Ca^{2+} activities, including Ca^{2+} spark, field stimulation-stimulated Ca^{2+} transient, SR Ca^{2+} load, and RyR2-mediated diastolic Ca^{2+} leak, were recorded by the LSM510 Meta inverted confocal microscope (Carl Zeiss) with a 40 ×/1.2 N.A water immerse objective. Cardiomyocytes were incubated with 10 μM Fluo-4 AM, a cell-penetrating Ca^{2+} indicator with Ca^{2+} binding affinity (K_d) of ~335 nM, at 37 °C for 5 min. Then cells were washed and kept in fresh bath solution. Binding to Ca^{2+}, Fluo-4 presents an increase in fluorescence, which is excited at the wavelength of 488 nm and recorded at wavelength > 505 nm. Ca^{2+} images were collected by the one-direction line scan of the long axis of cell, at the speed of 3.072 ms/line. For β-adrenergic stimulation, 300 nM isoproterenol was applied in bath solution. Ca^{2+} sparks, RyR2-mediated diastolic Ca^{2+} leak, Ca^{2+} transients, SR Ca^{2+} load, SERCA rate and NCX rate were measured as described in data supplement. For simultaneous recording of action potential and Ca^{2+} transient, cardiomyocytes were dialyzed with 0.2 mM fluo-4 pentapotassium salt via pipette solution. Ca^{2+} transients were recorded by the Olympus IX51 inverted microscopy system with a 40 × oil immerse objective.

2.4. Patch clamp

Whole-cell patch clamp experiments were conducted by using an Axopatch 700B and a Digidata 1440A digitizer (Axon Instruments) at room temperature. I_{Ca}, I_{NCX}, Action potential and action potential-triggered Ca^{2+} transient were measured as described in data