Junctophilin-2 Expression Silencing Causes Cardiocyte Hypertrophy and Abnormal Intracellular Calcium-Handling

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Background—Junctophilin-2 (JPH2), a protein expressed in the junctional membrane complex, is necessary for proper intracellular calcium (Ca\(^{2+}\)) signaling in cardiac myocytes. Downregulation of JPH2 expression in a model of cardiac hypertrophy was recently associated with defective coupling between plasmalemmal L-type Ca\(^{2+}\) channels and sarcoplasmic reticular ryanodine receptors. However, it remains unclear whether JPH2 expression is altered in patients with hypertrophic cardiomyopathy (HCM). In addition, the effects of downregulation of JPH2 expression on intracellular Ca\(^{2+}\) handling are presently poorly understood. We sought to determine whether loss of JPH2 expression is noted among patients with HCM and whether expression silencing might perturb Ca\(^{2+}\) handling in a prohypertrophic manner.

Methods and Results—JPH2 expression was reduced in flash-frozen human cardiac tissue procured from patients with HCM compared with ostensibly healthy traumatic death victims. Partial silencing of JPH2 expression in HL-1 cells by a small interfering RNA probe targeted to murine JPH2 mRNA (shJPH2) resulted in myocyte hypertrophy and increased expression of known markers of cardiac hypertrophy. Whereas expression levels of major Ca\(^{2+}\)-handling proteins were unchanged, shJPH2 cells demonstrated depressed maximal Ca\(^{2+}\) transient amplitudes that were insensitive to L-type Ca\(^{2+}\) channel activation with JPH2 knockdown. Further, reduced caffeine-triggered sarcoplasmic reticulum store Ca\(^{2+}\) levels were observed with potentially increased total Ca\(^{2+}\) stores. Spontaneous Ca\(^{2+}\) oscillations were elicited at a higher extracellular \([Ca^{2+}]\) and with decreased frequency in JPH2 knockdown cells.

Conclusions—Our results show that JPH2 levels are reduced in patients with HCM. Reduced JPH2 expression results in reduced excitation-contraction coupling gain as well as altered Ca\(^{2+}\) homeostasis, which may be associated with prohypertrophic remodeling. (Circ Heart Fail. 2011;4:214-223.)

Key Words: JPH2 ■ junctophilin ■ hypertrophic cardiomyopathy ■ calcium ■ ryanodine receptor

Junctophilins are members of a family of proteins found in all excitable cells from striated muscle to neurons that bridge the subcellular space between the plasma membrane and the sarco/endoplasmic reticulum (SR/ER). In cardiac tissue, JPH2-encoded junctophilin type 2 (JPH2) plays a critical role in maintaining effective calcium (Ca\(^{2+}\)) flux. In cardiocytes, voltage-gated L-type Ca\(^{2+}\) channels (LTCC) at the sarcolemma allow for an influx of extracellular Ca\(^{2+}\), which triggers Ca\(^{2+}\) release from the SR via intracellular Ca\(^{2+}\) release channels known as ryanodine receptors (RyR2). This process, known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), regulates myocyte contraction. Contraction is terminated, and relaxation is initiated, by uptake of cytosolic Ca\(^{2+}\) back into the SR through the action of the SR Ca\(^{2+}\) ATPase (SERCA2)

or transport into the extracellular space by the sodium-calcium exchanger (NCX1). A growing body of evidence suggests that defects in the communication between LTCC and RyR2 play a central role in the development of hypertrophic cardiomyopathy (HCM) and heart failure.

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HCM, defined as unexplained cardiac hypertrophy, affects approximately 1 in 500 persons and is one of the most common genetic cardiovascular diseases. In addition, HCM is the most common cause of sudden cardiac arrest in young athletes and a significant cause of sudden death in the young in general. Classically, HCM is viewed as a disease of the cardiac sarcomere whereby mutations in the genes encoding key sarcomeric proteins of the heart, such as MYH7-encoded
β-myosin heavy chain (MYH7) and MYBPC3-encoded cardiac myosin-binding protein C (MYBPC3), cause pathological cardiac hypertrophy. Whereas the pathogenesis of HCM may be initiated with a genetic mutation, emerging evidence has associated gain-of-function disruptions in Ca\(^{2+}\) homeostasis that increase intracellular Ca\(^{2+}\) levels with induction of a prohypertrophic response. Increased Ca\(^{2+}\) release from the SR, either through impaired inhibition of RyR2 function, or increased expression and function of SR inositol 1,4,5-trisphosphate receptors (IP3Rs), have been associated with cardiac hypertrophy.\(^9\),\(^10\) Furthermore, activation of IP3Rs in the nuclear envelope and increased store-operated Ca\(^{2+}\) entry through the transient receptor potential protein TRPC3 can similarly lead to cardiac enlargement in animal models.\(^11\),\(^12\) In some patients with HCM, unabated disease progression results in further pathogenic remodeling, ultimately leading to heart failure with loss of contractility.\(^13\)

Heart failure is one of the major causes of morbidity and mortality in the United States as well as the world.\(^14\) Depressed contractility, particularly rate-dependent contractility reserve, is a common feature of a failing myocyte, and several lines of evidence point to an underlying defect in Ca\(^{2+}\) handling.\(^15\) Compared with gain-of-function defects in HCM, failure generally demonstrates a loss-of-function defect in CICR, prolonged transient duration, and increased basal Ca\(^{2+}\) levels.\(^16\) Loss of CICR is likely because of the uncoupling of RyR2 from the LTCC, either functionally or physically, resulting in an orphaned RyR2 that has reduced sensitivity to Ca\(^{2+}\) entry from the LTCC.\(^4\) This is reflected in a loss of excitation-contraction (e-c) coupling gain, such that the same influx of extracellular Ca\(^{2+}\) produces a reduced RyR2-mediated Ca\(^{2+}\) transient.\(^17\),\(^18\) Despite the apparent dichotomous association of gain-of-function Ca\(^{2+}\) defects in HCM and loss-of-function Ca\(^{2+}\) defects in heart failure, there is evidence for overlapping Ca\(^{2+}\) perturbations between the disease states.

JPH2, the major cardiac junctophilin isoform, is necessary for the critical approximation of the LTCC with the RyR2 in the dyad.\(^19\),\(^20\) Amino-terminal membrane occupation and recognition nexus (MORN) motifs interact with the sarclemma, whereas a carboxy-terminal transmembrane domain tethers the opposite end to the SR. The critical role of this protein in maintaining the precise subsarcolemma geometry needed for CICR is underscored by the embryonic lethality of JPH2-deficient mice stemming from altered dyadic ultrastructure and stochastic Ca\(^{2+}\) transients with reduced amplitude.\(^19\) Loss of JPH2 expression was observed in rodent models of HCM and heart failure and is present during an early stage of dyadic “intermolecular failure.”\(^21\),\(^22\) In skeletal muscle, JPH expression silencing resulted in loss of triadic ultrastructure and reduced CICR amplitude.\(^23\) Recently, we identified three patients with HCM hosting mutations in JPH2, overexpression of which reduced CICR and disrupted cellular ultrastructure.\(^24\)

To this end, we sought to determine whether a loss of JPH2 protein expression is found in human HCM, and to determine the effect of JPH2 gene silencing on Ca\(^{2+}\) handling within the cardiocyte. We demonstrate reduced JPH2 expression in patients with HCM as well as increased HL-1 cell size and upregulation of the prohypertrophic gene program with expression silencing. Further, our observation that maximal Ca\(^{2+}\) transient, caffeine-stimulated store Ca\(^{2+}\) release, and spontaneous transients at low extracellular Ca\(^{2+}\) levels were each reduced, supporting a conclusion that JPH2 knockdown may result in reduced e-c coupling through uncoupled RyR2 and LTCC.

Methods

Human Cardiac Tissue

Human cardiac tissue was obtained from the left ventricle of traumatic death victims with no structural heart disease (BioChain Institute, CA; P1234139, Lot AS6041; P1234138, Lot A710149; and CP-R01-T1234130, Lot B201244). Left ventricular myectomy tissue samples from patients with HCM were obtained according to protocols approved by the Mayo Foundation Institutional Review Board following informed consent. Immediately on surgical resection, tissue samples were flash frozen in liquid nitrogen and stored at −80°C. Each HCM specimen was genotyped comprehensively for mutations in MYH7, MYBPC3, MYL2-encoded regulatory myosin light chain, MYL3-encoded essential myosin light chain, TNN1-encoded troponin I, TPM1-encoded α-tropomyosin, TNNT2-encoded troponin T, and ACTC-encoded α-cardiac actin to determine the genetic substrate for HCM pathogenesis using previously outlined methods.\(^25\) Although not equivalent to the clinical genetic testing panel, these genes contain the majority of HCM-associated mutations.

Western Blot

Western blot analysis on whole protein lysates was conducted as previously described.\(^26\) In brief, reduced lysates were resolved by SDS-polyacrylamide gel electrophoresis (4% to 15% for JPH2, CAV3, GAPDH; 6% for RyR2; 7.5% for LTCC; 10% for NCX1, SERCA2; and 15% for PLN), transferred to a nitrocellulose membrane, and blotted with appropriate primary antibodies: anti-JPH2, anti-CAV3 (generous gift from Dr Jonathan Makielski), anti-LTCC (Alomone Labs, Jerusalem, Israel), anti-RyR2 and -PLN (Thermo Fisher Scientific, Barrington, IL), anti-NCX1 (Swant, Bellinzona, Switzerland), anti-SERCA2 (Santa Cruz Biotechnology, Santa Cruz, CA), and antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH, Millipore, Temecula, CA) antibodies. After application of appropriate secondary antibody, protein was detected using chemiluminescence reagent (PerkinElmer, Waltham, MA) or developed using Alexa-Fluor680-conjugated anti-mouse (Invitrogen Molecular Probes, Carlsbad, CA) and/or IR800Dye-conjugated anti-rabbit secondary antibodies (Rockland Immunocchemicals, Gilbertsville, PA), and scanned on an Odyssey infrared scanner (Li-Cor, Lincoln, NE). Band density was quantified using ImageJ software.\(^26\) For human myectomy studies, samples were run unblinded to genetic mutation status and each replication Western blot used a different portion of the subject’s myectomy tissue.

Short Hairpin RNA Construct, Cell Culture, Transfection, Adenovirus Infection

The shRNA-encoded construct used for transient transfection experiments was described previously.\(^27\) In brief, screening experiments for RNA interfering probes to suppress JPH2 transcription identified a probe that targeted a conserved region of the mRNA encoding the second MORN motif domain of JPH2 (shJPH2) and JPH1.\(^23\) Subsequent cloning of the oligonucleotide into the pcMS-EGFP (BD Biosciences Clontech, Mountain View, CA) vector, in which the GFP reporter was exchanged with DrRed2, created the transient transfection construct pcMS-shRNA.

Lipofectamine-based transfection of pcMS-shRNA maximally achieved a 1% transfection rate in our studies. Whereas suitable for single-cell analyses (cell size, Ca\(^{2+}\) transient and store studies), high-efficiency shRNA oligo delivery was obtained through viral transduction/infection in studies requiring 100% shRNA delivery.
Adenoviral delivery of shJPH2 and shLuc was achieved by insertion of the shRNA-encoding cassette into the Dual-Basic-RFP construct before ligation of the shRNA-RFP expression cassettes into a shuttle vector and in vitro recombinant to Ad5 adenoviral backbone with E1/E3 deletion (Vector Biolabs, Philadelphia, PA) creating AdX-shRNA. Virus was packaged in HEK293 cells to produce viral lysates, viral titer was measured by Adeno-X Rapid Titer Kit (Clontech Laboratories, Inc.), and the viral lysate was stored in 1 mol/L sucrose, 5% tertiary amine beta cyclodextrin in PBS (in mM: 137 NaCl, 10 NaH2PO4, 2.7 KCl, 1 KH2PO4, pH 7.4).23

Murine atrial tissue-derived HL-1 cells were maintained as described previously.24 In brief, low passage number HL-1 cells were grown on 0.02% gelatin, 1% fibronectin-coated surfaces with Claycomb media (10% FBS [Sigma, Lot 116K8400], 100 U/mL pen, 100 μg/mL strep, 0.1 mmol/L norepinephrine, 2 mmol/L L-glutamine). For all confocal analysis, 5×10⁴ cells were plated on 23-mm-diameter glass cover slip Delta TPG culture dishes (Biotechps, Butler, PA). For Western blot analysis, 5×10⁵ cells were plated on 60-mm-diameter plastic tissue culture dishes (Falcon). Cells were transiently transfected using GeneJacker transfection reagent (Stratagene) per the manufacturer’s instructions with media replacement every 48 hours. Cells were assayed 96 hours posttransfection or infection and shRNA delivery was confirmed through DsRED2 or RFP fluorescence, respectively. Before phenylephrine (PE) treatment, cells were maintained in Claycomb media without norepinephrine for 96 hours to ensure absence of adrenergic receptor desensitization. Cells were then treated with Claycomb media containing 100 μmol/L PE (Sigma) or vehicle.

Cell Size and Calcium Measurements

Measurements of cell size and intracellular Ca²⁺ levels were performed using an Axiovert 200 mol/L-Apoptome (Carl Zeiss Inc., Santa Clara, CA) system with a rapid filter changer (Lambda DG-4, Sutter Instrument, Novato, CA), and analyzed using Axiovision v4.6 (Carl Zeiss Inc.) software. For all cell size experiments, the area of RFP-positive cells was measured by tracing the cell periphery after scaling calibration. To confirm accurate identification of the sarcolemma and accurate cell size measurements, wheat germ agglutinin (WGA, Invitrogen) diluted in Hank’s balanced salt solution (Invitrogen) was used to fluorescently label the sarcolemma, and cell size measurements were repeated in an independent assay. Before [Ca²⁺]ᵢ measurements, cells were washed twice with Tyrode buffer (in mM: 140 NaCl, 5 KCl, 10 HEPES, 2.5 CaCl₂, 2 MgCl₂, 10 d-glucose; pH 7.4), followed by loading for 45 minutes with 2.5 μmol/L Fura-2/AM or 5 μmol/L Fluo-4 AM dye (Invitrogen) before 15 minutes de-esterification in Tyrode. For Fura-2 AM studies, regular spontaneous Ca²⁺ transients were measured via fluorescence versus time with maximal Ca²⁺ transients (Fₘₐₓ) measured as the peak fluorescence achieved relative to basal fluorescence (Fₒ). Ratiometric 340/380 nm measurements allowed for correction of intracellular dye levels and estimation of rest basal Ca²⁺ levels. For Fluo-4 AM studies, transients were measured via fluorescence versus time. Transient amplitude (Fₘₐₓ/Fₒ), time to amplitude peak (Tₚₑᵃᵏ), and time to decay to 50% the maximal fluorescent intensity (T₅₀) were measured. Pharmacological modulation of LTCC activity was achieved by addition of two LTCC agonists: 10 μmol/L (S)−(-)-Bay K864420 (BayK; Tocris Bioscience, Ellisville, MO) and 500 nmol/L FPL 6417620 (FPL; Tocris Bioscience) and an LTCC antagonist: 100 nmol/L nifedipine40 (Tocris Bioscience) during Fluo-4 AM de-esterification. Alteration of extracellular Ca²⁺ levels ([Ca²⁺]₀) was achieved by equilibrating cells in Tyrode buffer with no Ca²⁺ ([Ca²⁺]₀) without EGTA buffering for 10 minutes, and successive increases in [Ca²⁺]₀ used similarly prepared Tyrode buffer with appropriate CaCl₂ concentrations.

To ascertain SR-stored Ca²⁺ levels through activation of RyR2, 10 mmol/L caffeine (Sigma) was applied to the Tyrode-immersed, Fura-2-loaded cells directly under real-time confocal analysis. To determine store Ca²⁺ levels through an SR-independent release mechanism, cells were treated with 10 μmol/L ionomycin (Sigma) which is a Ca²⁺ ionophore known to permeabilize membranes allowing for free movement of Ca²⁺.25 Before ionomycin treatment, cells were rapidly switched to 0Na⁺/0Ca²⁺ Tyrode (in mM: 140 NaCl, 5 KCl, 10 HEPES, 4.5 MgCl₂, 10 EGTA, 10 d-glucose, pH 7.4) to prevent influx of extracellular Ca²⁺ with treatment as well as alterations in cytosolic Ca²⁺ because of the action of the LTCC and the NCX1.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from HL-1 cells 96 hours after infection with AdX-shRNA using the RNeasy Protect Mini Kit (Qiagen) before storage at -80°C. RNA integrity was confirmed by Agilent 2100 Bioanalyzer analysis before DNA library generation utilizing poly-T primer-based reverse transcription (Quanta Biosciences, Gaithersburg, MD). RNA expression levels of skeletal actin (ACTA, Mm00808218_g1), atrial natriuretic factor (ANF, Mm01255747_g1), brain natriuretic factor (BNP, Mm00435304_g1), MYH7 (Mm00600555_m1), and regulator of calcineurin 1-exon 4 splice isoform (RCAN1-4, Mm00627762_m1) were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) via TaqMan Gene Expression Assays (Applied Biosciences, Foster City, CA). Detection of an interferon (IFN)-response by the cells was conducted by RNA expression levels of 2-5' oligoadenylate synthetase 1b (OAS1, Mm00449297_m1) and signal transducer and activator of transcription 1 (STAT1, Mm00439531_m1). The threshold cycle value, representing the number of PCR cycles (Cₚ) at which a normalized reporter signal (Rn) first crosses a detection threshold, was determined. The inverse level of RNA template was calculated and normalized to endogenous GAPDH (4352932E) levels. Relative fold change in expression of AdX-shJPH2 was then calculated compared to AdX-shLuc treatment.

Statistics

ANOVA and t test analysis was conducted for all studies, as appropriate, with the threshold of significance set to P<0.05. All statistical analyses were done on at least 3 independent experiments and are expressed as mean±standard error of the mean.

Results

JPH2 is Downregulated in Human Hypertrophic Cardiomyopathy

Previous investigations have shown JPH2 downregulation in rodent models of HCM, dilated cardiomyopathy, pressure-induced hypertrophy, and decompensated cardiac failure.21,22 Despite these findings, little is known about the expression levels of JPH2 in human disease. Surgical myectomy to remove left ventricular septal myocardium, a treatment for obstructive HCM, provides a relatively unique means of acquiring diseased myocardium from human subjects. Given this resource, we sought to determine whether the downregulation of JPH2 previously seen in rodent models of cardiac disease was also a characteristic of HCM in humans.

Western blot analysis of JPH2 expression levels from left ventricular protein lysates obtained from 3 otherwise-healthy traumatic death victims were compared with protein lysates obtained from 11 patients with HCM. Five were acquired from patients with mutations in MYH7 (hosting MYH7-G741R, -R663H, -I736T, -R403Q, and -M922K mutations from left to right lanes in Figure 1A) and three with mutations in MYBPC3 (MYBPC-M258K, -L527 fs/3, and -L1258 fs/71, respectively), which represent the two most common genetic subtypes of HCM. Further, 3 samples obtained from probands not hosting mutations in the analyzed genes were similarly assayed. Whereas there was some variation in JPH2 expression among presumably “healthy” subjects, a reduction in expression was observed in all samples acquired from pa-
tients with HCM. Quantification of blots taken from independent portions of resected myocardium revealed that all individuals with HCM had reduced JPH2 expression (ranging from 0.011 to 0.343 normalized intensity units) compared with trauma samples (from 1.536 to 0.700) as depicted in Figure 1B. When taken as a whole, HCM samples had a mean relative JPH2 expression of 0.120 compared with ostensibly healthy tissue (1.079, \( P = 0.0001 \); Figure 1B, inset).

shJPH2 Achieves Durable Expression Knockdown

Given the reduction of JPH2 protein expression in human HCM, we sought to determine whether this phenomenon was a compensatory physiological response of the heart or whether it represents a potential initiating factor in hypertrophic remodeling. To address this, interfering RNA targeted to JPH2 mRNA was introduced into HL-1 cells, which express endogenous JPH2 as well as all critical cellular components necessary for cardiac excitation contraction coupling, including RyR2, LTCC, SERCA2, NCX1, and the \( \beta \)-adrenergic receptor. These cells demonstrate spontaneous CICR and have been used previously to characterize the effect of genetic mutations in RyR2 function. Previous studies utilizing this shRNA probe have demonstrated a significant reduction in the expression levels of JPH1 as well as JPH2 in skeletal muscle; however, HL-1 cells do not express JPH1 by qRT-PCR (Figure 2A), leaving JPH2 as the only junctophilin family member silenced by expression of this shRNA.

Utilizing the RFP marker coexpressed by the adenovirus, we achieved nearly 100% infectivity of the HL-1 cells with a multiplicity of infection of 100 IFU/cell (data not shown). As shown in Figure 2B and C, adenoviral delivery of the shJPH2 probe demonstrated a sustained JPH2 expression knockdown of 26.5% after 96 hours when normalized to GAPDH expression relative to shLuc (\( P = 0.01 \)). The expression of caveolin-3 (CAV3), a JPH2 binding partner, was not affected by JPH2 silencing.

Expression of double-stranded RNA in mammalian cells may induce a type 1 IFN response that induces global alterations to multiple cellular processes, which may confound the effect of JPH2 expression silencing. To ensure that such a response was not present with adenoviral delivery of AdX-shJPH2 and shLuc, and to test this known cardiac remodeling signaling cascade, RNA expression levels of IFN-induced OAS1 and STAT1 were determined by qRT-PCR. As demonstrated in Figure 2D, there was no alteration in OAS1 and STAT1 levels in HL-1 cells treated with AdX-shJPH2 relative to controls normalized to GAPDH expression.

Figure 2. Adenovirus-mediated shRNA silencing of JPH2 in HL-1 cells. A, Representative qRT-PCR plot of normalized fluorescence (Rn) intensity versus PCR cycle number (C\(_T\)) for three transcript-specific probe sets including JPH1, JPH2, and GAPDH targeted to untreated HL-1 cDNA. B, Western blot of JPH2, CAV3, and GAPDH levels from HL-1 cells infected with AdX-shLuc and controls. C, Bar graph showing relative difference in band density of Adx-shJPH2 infected cells from independent Western blots relative to Adx-shLuc normalized to GAPDH. D, Bar graph demonstrating mean RNA expression levels of OAS1 and STAT1 by qRT-PCR of Adx-shJPH2 infected cells relative to controls (\( n = 6 \) runs).
JPH2 Knockdown Increases Cell Size and Induces Prohypertrophic Marker Expression

To determine whether an acute suppression of JPH2 expression is sufficient to initiate cardiac hypertrophic remodeling, HL-1 cell area was measured after both transient transfection and adenoviral infection of shJPH2. In pCMS-JPH2 transfected cells, partial JPH2 expression silencing resulted in a marked increase in HL-1 cellular size of 75.3±7.5% (n=68) compared with control shLuc cells (n=245; P<0.0001). To confirm accuracy of cell size measurements, we fluorescently labeled the cell sarcomella with WGA (Figure 3A) and confirmed an increase in cellular size of 57.0±14.0% (n=59) compared with control (n=80; P=0.004) which was not statistically significantly different from the degree of hypertrophy seen without WGA-labeling (P=0.2). Similarly, adenoviral delivery of shJPH2 resulted in an increase of 94.2±7.3%, n=346, compared with AdX-shLuc (n=469; P<0.0001) which was not statistically significant between transfection delivery of shJPH2 (P=0.3, Figure 3B). As a positive control, a known pharmacological inducer of hypertrophy in this cell line, PE, was applied (Figure 3B, light-gray bars). Treatment of these cells with an α1-adrenergic receptor agonist initiated a similar hypertrophic response (increase of 75.4±7.9%, n=485) relative to vehicle treated cells (n=657; P<0.0001) compared with shJPH2 expression silencing.

Because many potential pathways could contribute to increased cellular size in vitro, we attempted to correlate this observation with molecular markers of the well-established cardiac hypertrophic remodeling process. qRT-PCR was conducted on cells infected with AdX-shRNA to test for induction of several known markers of pathological hypertrophy. As shown in Figure 3C, a significant increase in ACTA (12.8±5.6%), ANF (10.6±4.4%), BNP (17.9±5.0%), and MYH7 (26.4±9.1%) was observed in AdX-shJPH2 cells compared with control cells (P<0.05). Furthermore, we tested transcriptional expression of RCAN1-4, a marker of calcineurin-dependent signaling which is one potential mediator of remodeling in the cardiocyte. In AdX-shJPH2-infected cells, there was no increase in RCAN1-4 expression levels by qRT-PCR.

JPH2 Knockdown Perturbs Calcium Flux and Homeostasis

Based on the observation of increased cellular area as well as transcriptional upregulation of prohypertrophic markers in the cells, we explored the possibility that Ca2+ dysregulation may be associated with JPH2 expression knockdown. Alterations in Ca2+ transients and basal Ca2+ levels have been linked to both the initiation of hypertrophy as well as arrhythmogenesis in the heart. HL-1 cells spontaneously and regularly oscillate Ca2+ through the action of e-c coupling proteins. We have previously used HL-1 cells to measure Ca2+-transient amplitude, and the firm adherence of these cells to the growth surface is suitable for maintaining real-time confocal measurements during pharmacological treatment.

Maximal Ca2+ transients achievable were significantly reduced in cells transiently transfected with pCMS-shJPH2 (1.10±0.09, n=69) compared with control cells (1.60±0.03, n=126; P<0.0001) as demonstrated in Figure 4A and B. This reduction in transient amplitude was insignificant to pharmacological stimulation of the LTCC. Whereas BayK and FPL, 2 LTCC stimulators, increased control cell transient amplitudes (1.75±0.07, n=43; P=0.008 and 1.88±0.07, n=53; P<0.0001 versus untreated control cells, respectively), this increase was not seen in shJPH2 cells (Bay K: 1.20±0.04, n=43 and FPL: 1.14±0.03, n=38) which demonstrated transient amplitudes that were not significantly different from untreated shJPH2 cells (Figure 4B). Conversely, application of nifedipine reduced Ca2+ transient amplitude in control cells (1.14±0.03, n=52; P<0.0001 versus untreated control cells), whereas there was no statistically significant difference between nifedipine-treated shJPH2 cells (1.06±0.02, n=28) and untreated cells (P=0.5, Figure 4B).

In addition to reductions in transient amplitude, transient kinetics were altered with JPH2 knockdown. Whereas the time to transient peak was unchanged, the time for the Ca2+...
transient to decay was reduced in shJPH2 cells (508 ± 46 ms, n = 49) compared with control cells (888 ± 49 ms, n = 119, Figure 4C and 4D). Despite impaired Ca\(^{2+}\) transient ampli-
tude, the resting Fura-2 AM fluorescence was higher in cells with shJPH2 (0.63 ± 0.03, n = 64) compared with control (0.55 ± 0.04, n = 47), although this finding was not statistically significant (P = 0.06, Figure 4E).

**JPH2 Knockdown Alters Store Calcium Levels**

To determine whether the reduction in Ca\(^{2+}\) transient and potential alteration in basal Ca\(^{2+}\) levels was because of alterations in store Ca\(^{2+}\), caffeine was applied to measure SR store Ca\(^{2+}\) levels. As demonstrated in Figure 5A and 5B, HL-1 cells transiently transfected with pCMS-shJPH2 had a significantly lower caffeine-induced Ca\(^{2+}\) release (0.82 ± 0.07, n = 24) compared to control (1.06 ± 0.05, n = 48; P < 0.0001). To determine whether this apparent loss of store Ca\(^{2+}\) was because of alterations in the SR Ca\(^{2+}\) store levels in isolation or whether other Ca\(^{2+}\) stores are similarly altered, ionomycin was applied. As demonstrated in Figure 5C and 5D, under 0Na\(^+\)/0Ca\(^{2+}\) buffer conditions, ionomycin treatment resulted in release of store Ca\(^{2+}\), which was greater in the pCMS-shJPH2 transfected cells (1.84 ± 0.10, n = 27) compared with controls (1.35 ± 0.06, n = 75; P < 0.0001). Thus, despite an overloaded total Ca\(^{2+}\) store in the shJPH2 cells, there was a significant reduction in caffeine-mediated SR store levels.

**JPH2 Knockdown Suppresses Spontaneous Calcium Oscillations**

The HL-1 cell line demonstrates regular spontaneous Ca\(^{2+}\) oscillations under normal extracellular [Ca\(^{2+}\)]. Based on the previously observed loss of Ca\(^{2+}\) transient amplitude and insensitivity of the Ca\(^{2+}\) transient to LTCC stimulation with JPH2 expression silencing, we next tested the coupling of spontaneous Ca\(^{2+}\) transients generation with extracellular [Ca\(^{2+}\)]. As demonstrated in Figure 6A, AdX-shLuc cells in 0Ca\(^{2+}\) buffer did not generate Ca\(^{2+}\) oscillations and occasionally demonstrate stochastic transients that presumably are not LTCC-triggered Ca\(^{2+}\) release events. With the addition of

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**Figure 4.** JPH2 knockdown reduces intracellular Ca\(^{2+}\) transients. A, Representative Ca\(^{2+}\) transient tracing of an HL-1 cell transiently transfected with pCMS-shJPH2 or shLuc measuring relative fluorescent intensity (F\(_{\text{max}}\)/F\(_{\text{o}}\)) versus time. B, Bar graph of the mean Ca\(^{2+}\) transient amplitude in HL-1 cells transiently transfected with pCMS-shJPH2 or control. Cells were treated with LTCC activators BayK or FPL or LTCC blocker nifedipine (Nif). Numbers in bar graphs indicate number of cells analyzed. **P < 0.01; ***P < 0.0001. C and D, Bar graph of time to transient peak and time to decay to half-maximum intensity, respectively, in HL-1 cells transiently transfected with pCMS-shJPH2 or control. ***P < 0.0001 E, Bar graph of the mean resting Ca\(^{2+}\) levels in cells transiently transfected with pCMS-shJPH2 or control. Numbers in bar graphs indicate number of cells analyzed.

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**Figure 5.** Knockdown of JPH2 increased store Ca\(^{2+}\) yet reduced RyR2-dependent caffeine-mediated store Ca\(^{2+}\) release. A, Representative Ca\(^{2+}\) tracing of HL-1 cell following caffeine application. B, Bar graph showing mean amplitude of Ca\(^{2+}\) efflux from the SR in pCMS-shRNA-treated cells exposed to caffeine. Numbers in bar graph indicate number of cells analyzed. ***P < 0.0001. C, Representative Ca\(^{2+}\) tracing of HL-1 cells following ionomycin treatment. D, Bar graph showing mean amplitude of Ca\(^{2+}\) efflux from all stores in pCMS-shRNA-treated cells exposed to ionomycin. Numbers in bar graph indicate number of cells analyzed. ***P < 0.0001.
0.1 and 0.25 mmol/L [Ca\(^{2+}\)]\(_o\), the stochastic, non-LTCC-mediated transients begin to yield regular oscillations that are coordinated among the cells. Application of 1 and 2.5 mmol/L [Ca\(^{2+}\)]\(_o\) induced regular oscillations that are spontaneous and are simultaneously triggered in nearly every cell in each field. In contrast, knockdown AdX-shJPH2 infected HL-1 cells do not demonstrate Ca\(^{2+}\) oscillations until 1 mmol/L [Ca\(^{2+}\)]\(_o\). This is quantified in Figure 6B, in which there is a blunting of Ca\(^{2+}\) oscillation generation with JPH2 silencing with 0.1 and 0.25 mmol/L [Ca\(^{2+}\)]\(_o\). This is reflected in a lower proportion of oscillating cells infected with AdX-shJPH2 than control at 0.1 mmol/L (0.04±0.01%, n=12 fields versus 0.13±0.02%, n=12; P=0.002) and 2.5 mmol/L (0.05±0.05%, n=13 versus 0.38±0.01%, n=11; P=0.009). Further, whereas there is no difference in the number of field-wide Ca\(^{2+}\) oscillations per time at lower [Ca\(^{2+}\)]\(_o\) levels, there is a significant reduction in the oscillation frequency in cells infected with shJPH2 (7.7±1.2 transients/30 s, n=15 fields) than shLuc (17.0±4.3, n=11 fields; P=0.02) at 2.5 mmol/L [Ca\(^{2+}\)]\(_o\), (Figure 6C).

**JPH2 Knockdown Does Not Alter Calcium-Handling Protein Expression**

Because of the observed reduction in Ca\(^{2+}\) transient amplitude, altered transient kinetics, and perturbed store Ca\(^{2+}\) levels, we next explored the expression levels of multiple proteins necessary for effective e-c coupling and transient formation (Figure 7). Despite the reduction of CICR amplitude, Western blot analysis of LTCC and RyR2 consistently revealed no change in expression with AdX-shJPH2-treated cells. Further, there was not a statistically significant change in expression of NCX1, SERCA2 and PLN in JPH2 knockdown cells compared to controls.

**Discussion**

The junctophilin family of proteins is critical for proper Ca\(^{2+}\) flux and homeostasis in excitable cells; including skeletal muscle (JPH1 and 237,38), cardiac muscle (JPH2), and neuronal tissue (JPH3 and 439–41). In concert with the recent insight into the cellular role of these proteins, genetic mutations that perturb the function of JPH2 and JPH3 have been associated with the pathogenesis of human disease. Although its pathogenic mechanism remains elusive, a CAG/CTG nucleotide repeat expansion in a potential alternatively spliced exon of JPH3-encoded junctophilin type 3 has been associated with Huntington disease type 2 in humans.42 Furthermore, we recently demonstrated 3 missense mutations in a cohort of 388 patients with HCM that were mutation negative for the traditional HCM-associated sarcomeric protein genes.24 Based on the emerging role of JPH dysregulation and dysfunction, we sought to understand how reduced expression of JPH2 might play a role in cardiomyopathy.

Herein, we demonstrated that myocardium from HCM patients demonstrates a significant reduction in JPH2 expression independent of underlying disease-causing genotype and may reflect a common feature in the cardiomyopathic remod-
eling of this disease. This fits well with the previous observations that reduction of JPH2 levels occurs across a relatively diverse range of rodent cardiomyopathic disease states. The generalizability of this phenomenon in human heart failure, pressure (hypertension)-induced hypertrophy, or myopathic sequelae of organic disease states such as idiopathic cardiomyopathy of diabetes, is an unanswered question. It is also possible that the downregulation of JPH2 is not a property of other mechanisms of cardiac hypertrophy.

To test whether this reduction of JPH2 expression is sufficient to induce cardiac dysfunction and hypertrophy, we used a RNA-silencing probe to knockdown JPH2 expression. Our observation that interfering RNA silencing of endogenous JPH2 results in a marked increase in cellular size, potentially through further induction of the fetal gene program, are evidence that even relatively small reductions in JPH2 expression can be an initiating factor in the pathogenic remodeling of the heart and not simply an adaptive response with no pathophysiologic consequence. In experiments using native cardiac tissue, upregulation of ACTA, ANF, BNP, and MYH7 as transcriptional markers of the prohypertrophic cellular response are on a fold-scale that is greater than the 10% to 30% increase we observe. As the HL-1 cells are immortalized and perpetually grow and divide, it is possible that there is a higher basal level of these transcripts within the transcriptional milieu of the cell, and we are observing a relative modest increase in a highly active progrowth/hypertrophic gene program. Alternatively, it is possible that the observed increase in cell size seen may not be solely mediated through the traditional hypertrophic response of which these transcripts are markers.

Silencing of JPH2 in HL-1 cells resulted in significant alterations in both basal and dynamic Ca\(^{2+}\) signaling within the cell. Our observations that JPH2 expression silencing results in reduced CICR through a reduction in the maximal transient amplitude without alteration of LTCC, RyR2, SERCA2, PLN, or NCX1 expression supports a role for JPH2 in maintaining effective e-c coupling gain. This closely follows our previous work in which functional characterization of the 3 HCM-associated mutations demonstrated a similar decrease in CICR amplitude when expressed in HL-1 cells and the reduced CICR seen in skeletal muscle with JPH1 and JPH2 knockdown. When Ca\(^{2+}\) is titrated into the extracellular buffer of spontaneously oscillating HL-1 cells, JPH2 knockdown reduces the sensitivity of CICR to extracellular Ca\(^{2+}\). Whereas RyR2-mediated Ca\(^{2+}\) transients are clearly present at 0.25 mmol/L [Ca\(^{2+}\)\(_{i}\)], with control shLuc-infected cells, no Ca\(^{2+}\) oscillations are seen in the shJPH2-infected cells. Because the cells did not demonstrate Ca\(^{2+}\) oscillations in 0Ca\(^{2+}\) extracellular buffer and at 2.5 mmol/L [Ca\(^{2+}\)]\(_{i}\), all cells regularly and uniformly oscillated in unison, extracellular Ca\(^{2+}\) entry through the voltage-sensitive LTCC is likely triggering the observed transients. Thus, it may be that the observed right shift in the dose–response curve with JPH2 knockdown is because of a loss of e-c coupling gain such that more Ca\(^{2+}\) entry into the subsarcolemmal space is needed to trigger sufficient RyR2 opening to cause CICR. This possibility is supported by the relative insensitivity of JPH2 knockdown Ca\(^{2+}\) transients to LTCC activation.

These loss-of-function defects in Ca\(^{2+}\) handling would generally reflect those seen in patients and experimental models of heart failure. Indeed, the loss of CICR amplitude, potential blunting of e-c coupling gain, and the observed reduction in SR store Ca\(^{2+}\) by caffeine stimulation would fit with several previous models of heart failure such as rats, dogs, and humans. Based on these previous studies, and the known role of JPH2 in maintaining critical dyadic geometry, one possibility is that this reduction in CICR may be because of the increased distance of RyR2 from the sarcoplasmal LTCC, or otherwise disrupted cellular ultrastructure. Although not directly addressed herein, this physical separation may result in a relatively Ca\(^{2+}\)-insensitive RyR2 that does not respond fully to Ca\(^{2+}\) influx from the LTCC, resulting in a loss of CICR. This possibility is supported by a previous observation that overexpression of HCM-associated JPH2 mutations disrupted cellular ultrastructure. In addition to the observed defect in CICR, alterations in cardiac relaxation are often seen in cardiac hypertrophy and failure. Reduced SERCA2 expression is commonly seen in models of cardiac hypertrophy and failure resulting in reduced SR Ca\(^{2+}\) and increased basal cytosolic Ca\(^{2+}\). Our observation that SERCA2 and PLN expression is unchanged is not without precedent and may reflect variation in the way this cellular model undergoes hypertrophy with JPH2 expression silencing. Although the protein levels are unchanged, it is possible that the ATPase activity of SERCA2, and the inhibitory role of PLN, are nonetheless functionally impacted by post-translational modification resulting in the observed reduction in SR Ca\(^{2+}\).

The loss of JPH2 expression in rodent models of HCM, our previous association of JPH2 mutations in human HCM, and our observation of loss of JPH2 expression in patients with HCM would seemingly run counter to a solely loss-of-function defect in CICR activity. Indeed, the development of cardiac hypertrophy has largely been associated with increases in cytosolic Ca\(^{2+}\), whereby either elevation of global or dyadic Ca\(^{2+}\) levels induce the prohypertrophic fetal gene program through either a calmodulin kinase II- or calcineurin-mediated pathway, among others. In a setting of reduced CICR amplitude, we observed a strong trend toward increased basal Ca\(^{2+}\) in JPH2 knockdown cells. It is unlikely that the increase in resting Ca\(^{2+}\) is directly due to alterations in the Ca\(^{2+}\) transient kinetics because the amplitude and TD\(_{50}\) are both reduced, and it may indicate increased Ca\(^{2+}\) leakage of RyR2 at rest. This possibility is in agreement with previous work showing that RyR2 mutations that disrupt the calmodulin binding that normally reduces RyR2 Ca\(^{2+}\) release activity, lead to induction of hypertrophy as well as extra Ca\(^{2+}\) oscillations that elevate cytosolic Ca\(^{2+}\) levels. Whereas the ability of JPH2 to modulate the gating of RyR2 has not been directly demonstrated to date, skeletal muscle JPH1 modulates gating of RyR1 through a redox-sensitive direct interaction. Any role, either direct or indirect, that JPH2 may have in modulating RyR2 channel function, as well as the involvement of prohypertrophic signaling pathways, will require future study.

There are several limitations to our study. Although we observe a clear reduction in JPH2 expression in HCM
myectomy specimens compared to the control samples, we are unable to evaluate the possibility of regional differences in cardiac JPH2 expression. In particular, we are unable to ensure that the control samples were obtained from the same region of the left ventricular myocardium as the HCM myectomy specimens. In addition, whereas the HL-1 cell line has proven useful for our analysis, the immortalized nature of these cells, loss of native cardiocyte shape, and t-tubular ultrastructure limits our analysis and prevents direct measurement of the intermolecular coupling of dyadic proteins. Further, we are unable to determine how knockdown of JPH2 expression might affect the beating heart in vivo and whether important hallmarks of cardiac remodeling, such as fibrous replacement of myocardium in heart failure, might be present. Future studies will need to be done to further elucidate any potential role JPH2 may have in modulating RyR2 function and how perturbations in this protein might predispose to Ca\(^{2+}\)-mediated diseases of the heart.

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**References**


**CLINICAL PERSPECTIVE**

Hypertrophic cardiomyopathy (HCM), defined as clinically unexplained hypertrophy of the left ventricle, is a common cause of autopsy-positive sudden death in the young, particularly athletes. In addition to the predisposition to sudden death, HCM is characterized by phenotypic and genotypic heterogeneity, whereby hundreds of mutations localizing to dozens of genes have been implicated in the pathogenesis of a disease with highly variable expressivity. This scientifically unexplained heterogeneity reveals a need to explore unique means by which cardiocytes can undergo hypertrophy. We have implicated *JPH2*-encoded junctophilin type 2 as one of these rare and novel HCM-associated genes, and we sought to explore the function and potential HCM-association of this protein. Herein, we demonstrate that surgical myectomy tissue derived from individuals with obstructive HCM exhibit significant downregulation of *JPH2* protein expression and, in some cases, have no detectable *JPH2* protein. We further demonstrate that expression silencing of this protein in vitro results in cellular hypertrophy with induction of several known hypertrophic markers. Further, this expression knockdown perturbs calcium signaling in a cell in a manner similar to many mouse models of HCM and heart failure. Ultimately, *JPH2* may play a critical role within the cardiocyte, and when perturbed, either by inherited mutations as previously shown, or loss of normal expression levels, there is a resulting hypertrophic cellular phenotype. This potentially has significant implications for HCM if, indeed, *JPH2*-mediated cardiac remodeling plays a role in the development of human LV hypertrophy independent of the defective genetic substrate that serves as the mutation biomarker for an individual’s disease. If additional studies validate our findings that reduced *JPH2* expression induces pathological cardiocyte remodeling, then the exploration of this proremodeling pathway in patients with HCM and other cardiomyopathic disease will yield additional insight into the molecular pathogenesis of this heterogeneous disease. Such insight may inform novel therapeutic intervention for individuals with this genetically and phenotypically diverse disease.
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