Unfolded Protein Response Regulates Cardiac Sodium Current in Systolic Human Heart Failure

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Background—Human heart failure (HF) increases alternative mRNA splicing of the type V, voltage-gated cardiac Na⁺ channel α-subunit (SCN5A), generating variants encoding truncated, nonfunctional channels that are trapped in the endoplasmic reticulum. In this work, we tested whether truncated Na⁺ channels activate the unfolded protein response (UPR), contributing to SCN5A electric remodeling in HF.

Methods and Results—UPR and SCN5A were analyzed in human ventricular systolic HF tissue samples and human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs). Cells were exposed to angiotensin II (AngII) and hypoxia, known activators of abnormal SCN5A mRNA splicing, or were induced to overexpress SCN5A variants. UPR effectors, protein kinase R-like ER kinase (PERK), calreticulin, and CHOP, were increased in human HF tissues. Induction of SCN5A variants with AngII or hypoxia or the expression of exogenous variants induced the UPR with concomitant downregulation of Na⁺ current. PERK activation destabilized SCN5A and, surprisingly, Kv4.3 channel mRNAs but not transient receptor potential cation channel M7 (TRPM7) channel mRNA. PERK inhibition prevented the loss of full-length SCN5A and Kv4.3 mRNA levels resulting from expressing Na⁺ channel mRNA splice variants.

Conclusions—UPR can be initiated by Na⁺ channel mRNA splice variants and is involved in the reduction of cardiac Na⁺ current during human HF. Because the effect is not entirely specific to the SCN5A transcript, the UPR may play an important role in downregulation of multiple cardiac genes in HF. (Circ Arrhythm Electrophysiol. 2013;6:1018-1024.)

Key Words: heart failure ■ humans ■ PERK kinase ■ sodium channels

Human systolic heart failure (HF) is associated with decreased cardiac voltage-gated Na⁺ channel current, and these Na⁺ channel changes have been implicated in the increased risk of sudden death in HF. The cardiac Na⁺ channel is a transmembrane protein composed of 4 homologous domains, each containing 6 transmembrane segments. SCN5A, encoding the α-subunit of the type V, voltage-gated cardiac Na⁺ channel, consists of 28 translated exons. Previously, we have shown that the Na⁺ channel mRNA is alternatively spliced, with 2 SCN5A splicing variants (E28C and E28D) increasing in HF because of an angiotensin II (AngII)- or hypoxia-mediated increase in the splicing factor complex RNA binding motif protein 25/LUC7L3 (RBM25/LUC7L3). These variants cause a reading frame shift, resulting in premature stop codons, and encode cardiac Na⁺ channels truncated before the pore-forming segment of domain IV of the channel. These variants cannot form functional channels and reduce Na⁺ current to a greater extent than their percentage of the total SCN5A mRNA (ie, a dominant negative effect). The mechanism of this dominant negative effect is unclear.

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The unfolded protein response (UPR) is a series of interrelated signaling pathways that occur when endoplasmic reticulum (ER) experiences excess secretory load, accumulates misfolded proteins, or is subject to other pathological conditions. UPR acts on several levels: it rapidly attenuates general protein synthesis, induces the expression of ER chaperone proteins, and enhances the degradation of misfolded proteins. These changes are presumably designed to restore protein folding and ER health. There seem to be 3 main sensor proteins that activate the UPR. Among these main ER stress sensors, protein kinase R-like ER kinase (PERK)-mediated UPR has been shown to be present in cardiomyocytes. In the heart, the UPR plays a role during development, hypertrophy, ischemia, and HF. HF is associated with hypoxia, elevated AngII, and increased catecholamines, all of which have been shown to activate the UPR. In this work, we investigated the role of the UPR in mediating the Na⁺ channel downregulation observed in HF.
Methods

Methods are described briefly below and reviewed in detail in the online-only Data Supplement.

Differentiation and Culture of Human Induced Pluripotent Stem Cells–Derived Cardiomyocytes (hiPSC-CMs)

The human iPS cell line, DF19-9-11T,\textsuperscript{21} was differentiated into CMs using the matrix sandwich method.\textsuperscript{22} CMs differentiated for 30 days were used in this study.\textsuperscript{22}

Human Heart Tissue Samples

Human heart tissue samples were composed of end-stage cardiomyopathy hearts (n=10) and nonfailing control hearts (n=6). Normal human ventricular tissue was kindly offered by Dr J. Andrew Wasserstrom (Northwestern University, Chicago, IL). The control RNA sample was obtained from Clontech (Mountain View, CA). Samples were analyzed under University of Illinois at Chicago Institutional Review Board approval (Protocol 2009-0881).

Real-Time Polymerase Chain Reaction Quantification

Total RNA was isolated from cultured cells and human ventricular tissue using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit, respectively (Qiagen, Valencia, CA). The amplification conditions were a holding stage of 95°C for 20 minutes and 40 cycles at 95°C for 30 s and 60°C for 60 s.\textsuperscript{7}

Figure 1. Unfolded protein response activation in human heart failure (HF) tissue. A, The fold increase in mRNA abundances of PERK, CCAAT/enhancer-binding protein homologous protein (CHOP), and calreticulin in failing heart tissue (n=3) relative to controls are shown. All mRNA abundances are normalized to \(\beta\)-actin. B, Western blot quantification confirms the upregulation of PERK, CHOP, and calreticulin in human HF tissue. HF1, HF2, and HF3 represent 3 separate, representative HF tissue samples, respectively. Quantification is based on 3 replicated experiments. All protein levels are normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (*\(P<0.05\) when compared with control, which was a mixture of 3 patient samples).

Figure 2. Protein kinase R-like ER kinase (PERK) is upregulated in human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) with treatments increasing SCN5A variants. A, The mRNA abundance changes of PERK in AngII treated, and cardiomyocytes overexpressing truncation variants E28C or E28D versus normal control hiPSC-CMs are shown at 24 hours. mRNA abundances are normalized to \(\beta\)-actin (*\(P<0.05\) when compared with control, n=5 for each experimental group). B, Western blot quantification confirms the upregulation of PERK in the different treatment groups of hiPSC-CMs (*\(P<0.05\) when compared with control, a mixture of 3 samples; n=5 for each experimental group).
Transfection and Infection Assays
C-terminal green fluorescent protein (GFP)–tagged E28C and E28D variant constructs were transduced into hiPSC-CMs to overexpress E28C or E28D. An empty vector and a vector expressing only the fluorescent marker were used as controls.

Electrophysiology
Na+ channel currents were measured from hiPSC-CMs by using the whole-cell patch-clamp technique in the voltage-clamp configuration at room temperature.

Statistics
All data are presented as means and the 95% confidence intervals (95% CI). Means were compared using unpaired Student t test or ANOVA. Time-dependent variables were tested by repeated measures ANOVA. A probability value of P<0.05 was considered statistically significant. Box and whisker plots show the median, second quartile, and 1.5 interquartile ranges. Line graphs with error bars are used to compare changes with time among multiple quantitative variables.

Results
UPR Components Were Upregulated in Human HF
To assess UPR activation in human HF, the expressions of the major UPR pathway components were evaluated by Western blot and qPCR. Compared with normal human heart tissue, the relative mRNA abundances of PERK, CCAAT/enhancer-binding protein homologous protein (CHOP), and calreticulin were increased by 1.82-fold (95% CI 1.76, 1.88; P<0.001), 2.22-fold (95% CI 2.14, 2.29; P<0.001), and 2.47-fold (95% CI 2.39, 2.55; P<0.001) in HF tissue, respectively (Figure 1A). mRNA findings were correlated with protein expression by Western blots. Representative Western blots are shown in Figure 1B. Compared with the control group, Western blot quantification showed that PERK, CHOP, and calreticulin were increased by 1.63-fold (95% CI 1.60, 1.65; P<0.001), 1.86-fold (95% CI 1.83, 1.89; P<0.001), and 2.10-fold (95% CI 2.06, 2.13; P<0.001) in the HF tissue samples (based on 3 replications for each group).

SCN5A Splice Variants Activate UPR
We tested whether the presence of SCN5A splice variants could induce PERK. hiPSC-CMs were used because abnormal SCN5A splicing has not been shown to occur in species other than humans and primates.6 AngII and hypoxia are common pathogenic factors in HF.12 We have shown that each increases the SCN5A variants by upregulating the splicing complex RBM25/LUC7L3,7 and we used each as upstream

Figure 3. Protein kinase R-like ER kinase (PERK) is involved in the SCN5A variant–mediated downregulation of full-length SCN5A. A, Hypoxia, AngII (200 nmol/L), or overexpression of variant E28C or E28D constructs reduced full-length SCN5A mRNA. In each case, this reduction was inhibited by anti-PERK shRNAmir. Preinfection by scrambled shRNA had no effect on the SCN5A mRNA reduction by any treatment. Quantitative polymerase chain reaction measurements by 3 duplicates are shown at 24 hours in each treatment group and normalized by β-actin (*P<0.05 compared with control group, n=5 for each experimental group). PERK-mediated mRNA decay assays for SCN5A, Kv4.3, and transient receptor potential cation channel M7 (TRPM7) are shown in B–D, respectively. Control (closed squares), AngII-treated (200 nmol/L, inverted triangles), AngII-treated with preinfection by anti-PERK shRNAmir (closed diamonds), and AngII-treated with preinfection by scrambled shRNA (closed circles) groups are shown. mRNA was harvested for each group at 0, 6, 12, and 24 hours. The target genes were measured by qPCR and normalized to β-actin. The error bars in B–D represented SE. AngII treatment reduced SCN5A (P=0.019, n=5 for each experimental group; B) and Kv4.3 (P=0.042, n=5 for each experimental group; C) mRNA stability when compared with control while having no effect on TRPM7 (P=0.078, n=5 for each experimental group; D). Preinfection by pGIPZ lentiviral anti-PERK shRNAmir but not scrambled shRNA could prevent mRNA instability (P=0.027, n=5 for each experimental group).
stimuli to induce abnormal SCN5A mRNA splicing. The lack of abnormal splicing in rodents is because of a lack of upregulation of the RBM25/LUC7L3 splicing complex for unknown reasons.

hiPSC-CMs were divided into 4 experimental groups: untreated control, AngII treated (200 nmol/L), and SCN5A variant E28C and variant E28D overexpression groups. The AngII dose was chosen based on previous experiments showing a maximal effect on variant abundance.7 Cells were harvested from each experimental group at 48 hours, and total mRNA was extracted. The expression of PERK was examined by qPCR in each group. The results showed that PERK mRNA was increased in each experimental group compared with control, ranging from 2.5- to 2.6-fold depending on the group (P=0.012 for each group; Figure 2A). Western blot revealed that PERK was increased in each experimental group, ranging from 1.5- to 2.5-fold depending on the group (P=0.033 for each group). The representative Western blots and quantification (based on 3 replications for each group) are shown in Figure 2B. However, overexpression of full-length SCN5A did not upregulate PERK (Figure I in the online-only Data Supplement).

PERK Causes SCN5A Variant–Mediated Downregulation of the Full-Length SCN5A mRNA

Activated PERK phosphorylates eukaryotic Initiation Factor 2α (eIF2α), increasing mRNA instability by inhibiting ribosomal association.23,24 To test whether PERK activation affected SCN5A stability, hiPSC-CMs cells were divided into 3 experiment groups: AngII-treated cells, AngII-treated cells preinfected by pGIPZ lentiviral anti-PERK shRNA/mir, and AngII-treated cells preinfected by scrambled shRNA. AngII (200 nmol/L) treatment was given to all experiment groups for 48 hours until cell harvesting. The infection rate was >90%, evaluated by the ratio of GFP-positive cells (pGIPZ lentiviral infected cells) to total cells. PERK knockdown efficiency was 60.0% (95% CI 55.3%, 64.7%; P=0.017) at 48 hours, evaluated by Western blot (Figure II in the online-only Data Supplement). Compared with the AngII-treated group, the expression of the full-length SCN5A mRNA was increased by 1.42-fold (95% CI 1.40, 1.44; P=0.031) when cells were preinfected with anti-PERK shRNA. Scrambled shRNA did not change the AngII effect. The results indicated that AngII-mediated SCN5A mRNA downregulation was dependent on PERK (Figure 3A). Similar results were observed when hiPSC-CMs were transfected with variant E28C or E28D constructs rather than treated with AngII. Full-length SCN5A was increased by 1.54-fold (95% CI 1.49, 1.59; P=0.044) and 1.47-fold (95% CI 1.38, 1.56; P=0.042), respectively, after preinfected with anti-PERK shRNA at 48 hours. Again, scrambled shRNA did not prevent the effect of the variants.

The specificity of the PERK effect on the downregulation of full-length SCN5A was evaluated by analyzing mRNA stability of 2 other channels regulated by AngII, Kv4.3,25,26 and transient receptor potential cation channel M7 (TRPM7).22 These 2 channels were chosen as representatives because both are present in cardiomyocytes,25,26 and because Kv4.3 but not TRPM7 is known to be regulated in HF.27 hiPSC-CMs were divided into 4 experimental groups: control (untreated), AngII-treated (200 nmol/L) cells, AngII-treated (200 nmol/L) cells...
cells preinfected by pGIPZ lentiviral anti-PERK shRNA, and AngII-treated (200 nmol/L) cells preinfected by a vector containing scrambled shRNA. Except for the control groups, AngII treatments were given to all the experiment groups for 24 hours. mRNA was extracted at 0, 6, 12, and 24 hours after AngII treatment. Compared with the untreated cells, the expressions of both SCN5A (Figure 3B) and Kv4.3 (Figure 3C) were reduced ≈ 60%, 70%, and 70% at 6, 12, and 24 hours, respectively, when cells were treated with AngII or treated with AngII and preinfected by scrambled shRNA, whereas the expression of both SCN5A and Kv4.3 mRNAs were only reduced ≈ 20%, 30%, 30% at 6, 12, and 24 hours, respectively, when cells were treated with the anti-PERK shRNAmir, implying that the downregulation of PERK prevented mRNA decay of both SCN5A and Kv4.3. There was no statistical difference between the SCN5A and Kv4.3 mRNA decay curves before or after PERK inhibition (P = 0.108), suggesting that both ion channels mRNA abundances were affected equivalently by PERK activation. There is no statistical difference in TRPM7 mRNA (Figure 3D) between the different treatment groups and at the various time points (P = 0.075), suggesting that the PERK-mediated UPR showed some degree of specificity.

Truncated Na+ Channels Were Localized to the Endoplasmic Reticulum (ER)

To examine the subcellular localization of truncated Na+ channels, we overexpressed pEGFP-C1-SCN5A E28C and pEGFP-C1-SCN5A E28D in hiPSC-CMs (Figure 4A). Detailed information of both constructs is shown in Figure 4B. In each case, the truncation variant was linked to GFP at the 5′ end. Therefore, green immunofluorescence indicated the localization of truncated Na+ channel encoded by E28C or E28D. The transfected cells were studied at 48 hours and stained with the ER marker calreticulin (in red, Figure 4A). The merged images showed the colocalization of truncated Na+ channel variants with calreticulin. This pattern was distinct from the membrane localization of full-length SCN5A (Figure III in the online-only Data Supplement).

PERK Activation Reduces Na+ Current

The functional consequence of PERK-mediated destabilization of SCN5A mRNA was tested by measuring Na+ current in hiPSC-CMs. The cells were divided into 3 experimental groups: control, AngII-treated (200 nmol/L), and AngII-treated (200 nmol/L) cells preinfected by anti-PERK shRNA. Cells were used for measuring Na+ current at 48 hours. The representative current traces from

Figure 5. Protein kinase R-like ER kinase (PERK) mediates downregulation of Na+ currents after AngII treatment. A–C. The representative current traces without normalization for membrane surface area and normalized current–voltage curves are shown from control (◼), AngII-treated (●), and AngII-treated cells with anti-PERK shRNA (▲). D. Current–voltage curves using current density are based on 5 cells in each group. shRNA lentiviral particles had no effect on AngII-mediated Na+ channel regulation (P = 0.094, data not shown).

Figure 6. A summary of the effects of abnormal Na+ channel splicing in end-stage, systolic heart failure (HF). AngII and hypoxia are 2 regulators for RNA binding motif protein 25/LUC7 like 3 (RBM25/LUC7L3)–mediated SCN5A splicing regulation.7 HF is associated with an increase in Na+ channel mRNA variants resulting from splicing at cryptic splice sequences in the terminal exon of SCN5A (ie, exon 28). These variants encode nonfunctional cardiac Na+ channels. Variant levels reach greater than 50% of the total SCN5A mRNA.6,7 Abnormal mRNA splicing and variant-mediated UPR activation contribute to a decreased in full-length Na+ channel mRNA, protein, and current in HF.
each group are shown in Figure 5. The peak Na$^+$ current density at −30 mV in AngII-treated human cardiomyocytes was reduced by 62% (95% CI 58%, 66%; P=0.027) when compared with control cells. The effect of AngII on peak current was abrogated by the pretreatment of anti-PERK shRNA. Scrambled shRNA lentiviral particles had no effect on Na$^+$ channel current regulation by AngII (P=0.091, data not shown). As found previously, gating changes could not explain the magnitude of peak current changes seen (Table I and Figure IV in the online-only Data Supplement).6

**Discussion**

Recently, we reported that 2 SCN5A mRNA splicing variants are upregulated in end-stage, systolic human HF tissue. These variants reach greater that >50% of the total SCN5A mRNA and do not produce functional channels.6,7 The expression of these variants in cells stably expressing the full-length channel causes a dose-dependent reduction in the transcripts of full-length SCN5A, as well as the reduction in functional Na$^+$ current expression.6,7 Moreover, a mutation in a single allele of SCN5A mimicking a variant causes an 86% reduction in Na$^+$ current, >50% predicted reduction.6 These data suggest that the variants have a dominant negative effect on the full-length channel, but the underlying mechanism is unclear. We hypothesized that UPR might be responsible for the variant-initiated, dominant negative reduction in Na$^+$ current.

Our data show that UPR was activated in end-stage, systolic human HF. Although the human heart experiments do not establish which cell type is experiencing UPR activation, the cardiomyocyte experiments establish that UPR regulates Na$^+$ current in these cells. We show that truncated Na$^+$ channels, encoded by SCN5A variants, become trapped in the ER and activate the UPR. UPR activation resulted in decreased full-length Na$^+$ channel mRNA, protein, and current. Previously, we have shown that full-length, C-terminal GFP–tagged SCN5A targets to the sarcolemma and functions normally, and untagged variants cause a dose-dependent reduction in SCN5A transcript,6 making unlikely the possibility that UPR activation was related to GFP tagging of the variants. Moreover, elevated AngII and hypoxia, conditions that exist in HF,12 and are known to increase the SCN5A truncation variants,5,7 had similar effects on PERK activation, suggesting that variant induction may contribute to the UPR with these stimuli. Inhibition of PERK prevented full-length SCN5A mRNA degradation and Na$^+$ current reduction, suggesting that UPR activation is downstream of variant translation and required for the variant-mediated reduction in Na$^+$ current.

UPR activation is thought to cause translational inhibition affecting a significant number of, but not all, proteins. The spectrum of proteins affected is unknown as is the mechanism by which some proteins avoid UPR-mediated inhibition. In our case, we showed that AngII-induced PERK activation was responsible for degradation of mRNA encoding the K$^+$ channel α-subunit, K,4,3, which encodes the cardiac transient outward current. Because this K$^+$ channel is downregulated in HF or by AngII,27–32 it seems likely that a mechanism of this downregulation is UPR activation. It is possible that UPR activation explains, at least partially, the coregulation of K,4,3 and Na$^+$ channels observed in HF.33 Another AngII-regulated ion channel, TRPM7, was not affected by PERK regulation, confirming that UPR has certain selectivity. Although we did not measure currents of K,4,3 or TRPM7, the changes in mRNA are consistent with those seen in HF.25,32,33 Therefore, the results make it clear that UPR does not affect all protein expression equally, but the basis for selectivity between proteins is unknown.

In summary, this and our previous results6,7 suggest that the reduction in Na$^+$ current during systolic human HF results in part from abnormal SCN5A mRNA splicing to produce nonfunctional channels that become trapped in the ER and activate the UPR. UPR activation leads to destabilization of the remaining full-length SCN5A mRNA, exacerbating the reduction in Na$^+$ currents. Figure 6 shows a current hypothesis for the effect of HF on SCN5A mRNA handling based on this and previous work. Although we were not able to test the extent to which the Na$^+$ channel reductions mediated in this way contribute to arrhythmia because the splice variants occur only in humans,6 the changes in current were in the range known to contribute to arrhythmic risk. The broad nature of the UPR effect may contribute to the downregulation of other critical proteins in end-stage, systolic HF.

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**Disclosures**

S.C. Dudley is the inventor on patent applications: (1) SCN5A Splice Variants for Use in Methods Relating to Sudden Cardiac Death and Need for Implanted Cardiac Defibrillators, PCT/US2012/020564 and (2) SCN5A Splicing Factors and Splice Variants For Use in Diagnostic and Prognostic Methods, 13/291,826.

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**CLINICAL PERSPECTIVE**

Many ion channels and other proteins are downregulated in human heart failure (HF), and the decrease is thought to contribute to the arrhythmic risk. The mechanisms that cause these downregulations are unclear but, if elucidated, could allow for interventions to prevent sudden death. One of the ion channels downregulated in HF is the cardiac sodium channel. Previously, we had shown this was, in part, related to abnormal messenger RNA processing that resulted in prematurely truncated message and short, nonfunctional channels. In this work, we show these short channel forms are trapped in the endoplasmic reticulum and activate a cellular stress response mechanism known as the unfolded protein response (UPR). Part of the UPR is to reduce protein translation. We showed that activation of UPR resulted in further decrease in the sodium channel and caused a reduction in a potassium channel. These changes could be inhibited by blocking the activity of the UPR effector, protein kinase R-like ER kinase (PERK), resulting in the recovery of sodium current. In summary, sodium channels are downregulated in human HF; UPR likely contributes to the downregulation of proteins and the arrhythmogenic risk in human HF; and this risk would likely be improved by blocking the UPR effector, PERK. The sodium channel downregulation may contribute to proarrhythmic effects of sodium channel blocking antiarrhythmic drugs. The full extent of the effects of UPR in HF and the specificity of the process remain to be determined. UPR may become an important target for intervention in HF.
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SUPPLEMENTAL MATERIAL

Methods

Differentiation and culture of human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CMs)

Human iPS cells were cultured on Matrigel (growth factor reduced, BD Bioscience, Bedford, MA) in mTeSR1 medium (WiCell Institute, WI) as a monolayer. Cells were overlaid with a thin layer of Matrigel (0.5 mg /6-well plate) in mTeSR1 medium when reaching 80-90% confluency and grown for another 1-2 days in mTeSR1 medium until reaching 100% confluency, defined as day 0. Then, the mTeSR1 medium was replaced with RPMI 1640 medium (Life Technologies, Carlsbad, CA) plus B27 without insulin supplementation (Life Technologies) and containing Activin A (100 ng/mL; R&D Systems, Minneapolis, MN) and Matrigel for 24 h. After which, the medium was changed to RPMI1640 plus B27 without insulin supplemented with bone morphogenetic protein 4 (R&D Systems; Minneapolis, MN 5 ng/mL) and basic fibroblast growth factor (Invitrogen, Grand Island, NY; 10 ng/mL) for 4 days. After the growth factor treatment, the cells were maintained in RPMI plus B27 complete supplement medium. Confluent contracting cells were observed at day 7. The method we used to differentiate hiPSC-CMs generates > 90% cardiomyocytes.1,2 Since the endpoint measurement was cardiac sodium channels, expressed almost exclusively in cardiac myocytes, it is unlikely that other cell types present after differentiation would affect the results significantly.

Human heart tissue samples

Among 10 end-stage cardiomyopathic heart samples, six samples had ischemic myopathy, four samples had nonischemic myopathy. Seven and three samples were from males and females, respectively. The age range of sample donors was is 34 to 75
years old, and their average age was 59 years old. End-stage cardiomyopathic heart samples were obtained at the time of left ventricular assist device (LVAD) placement or cardiac transplantation. Specimens were obtained under Advocate Christ Medical Center Institutional Review Board (IRB) approval (Protocol 3184) from samples that would have been otherwise discarded. Subjects with end-stage cardiomyopathy exhibited severely reduced ejection fraction, left ventricular dilation, elevated pulmonary arterial and wedge pressures, and a reduced cardiac index. The control samples were obtained from donor hearts unused for transplantation. Control subjects were younger (median age 42 years with a range of 24–50 years) but with a similar gender profile (66% male). Samples were analyzed under University of Illinois at Chicago IRB approval (Protocol 2009-0881). The investigations conformed to the principles outlined in the Declaration of Helsinki.

**Real-Time PCR quantification**

Total RNA was isolated from cultured cells and human ventricular tissue using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit, respectively (Qiagen, Valencia, CA). Primers for target genes were: SCN5A (5’-TTACGCACCTTCCCAGTCCTCC-3’; 5’-GATGAGGGCAAGACGCTGAGG-3’); HSCN5AE28C/R (5’-TCTCTTCTCCCTCTCTGGTCA-3’); HSCN5AE28D/R (5’-GGAAGAGCGTGGGGAGAAGAAGTA-3’); PERK (5’-AGTCTCTGCTGGAGTCTTCATGACACTGTGTCTCAGACTTT-3’); Kv4.3 (5’-GCGCGCGAATTCCCTTTTGCCCGGGAGAAGATGA-3’); CHOP (5’-CCTAGCTTGGGCTGACAGAGA-3’; 5’-CTGCTCCTTCTCCCTTGCATGC-3’); calreticulin (5’-CATGATGGACATGATGATGACAC-3’; 5’-GGTCTTCAGACTTTCCCGGCTGC-3’); β-actin (5’-GGATCGGCGGCTCCAT-3’; 5’-CATACTCCTGCT-3’).
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TGCTGATCCA-3’). TRPM7 primers were obtained from the All-in-One™ qPCR kit (Genecopoeia, Rockville, MD). The amplification conditions were a holding stage of 95°C for 20 min and 40 cycles at 95°C for 30 s and 60°C for 60 s.

Transfection and infection assays

Human cardiomyocytes were placed in a 24-well plate at the density of 200,000/well. PERK short hairpin RNA (shRNA; 5 µL for each well based on pre-titer results) was pre-incubated with polybrene (Sigma, Milwaukee, WI) at final concentration 8 µg/mL for 1 h and aliquoted to each well. Fugene 6 reagents from Roche (Madison, WI) were used for transfection assays following the manufacturer’s instructions. Human pGIPZ lentiviral short hairpin RNA mir anti-PERK particles were purchased from Open Biosystems (Huntsville, AL). Human cardiomyocytes were placed in a 24-well plate at the density of 200,000/well. PERK short hairpin RNA (shRNA; 5 µL for each well based on pre-titer results) was pre-incubated with polybrene (Sigma, Milwaukee, WI) at final concentration 8 µg/mL for 1 h and aliquoted to each well. The scrambled shRNA group followed the same protocol. The media was replaced by regular culture media after 5 h. The infection rates and PERK knockdown rates were evaluated by Western blotting and quantitative PCR (qPCR) respectively on day 2 and day 3. C-terminal GFP-tagged E28C variant, E28D variant, or full-length SCN5A constructs were transduced into cells. Previously, we have shown that full-length, C-terminal GFP tagged channels have normal targeting and function.¹ An empty vector and a vector expressing only the fluorescent marker were used as controls. The transfection assays followed the manufacturer’s instructions.

Immunofluorescence staining and confocal microscopy
**SUPPLEMENTAL MATERIAL**

Transfected hiPSC-CMs on coverslips were fixed with 4% paraformaldehyde (with 4% sucrose in phosphate buffered saline (PBS)) for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 5% bovine serum albumin for 1 h. Fixed cardiomyocytes were then incubated with the primary antibody, anti-calreticulin (Abcam, Cambridge, MA), at room temperature for 1 h. After washing with PBS (3 X 5 min), the cells were incubated with Alexa Fluor 594-labeled secondary antibody (1:1000, room temperature 1 h). The cells were then washed with PBS (3 X 5 min) and mounted with ProLong Gold and stained with DAPI (4′,6-diamidino-2-phenylindole) (Life Technologies). CellMask Plasma Membrane Stains (Invitrogen) was used for cell plasma membrane staining. The detailed method followed manufactory protocol. Fluorescent imaging was acquired using a Carl Zeiss LSM 510 Meta confocal microscope.

**Electrophysiology**

hiPSC-CMs were trypsinized (0.25% trypsin-EDTA, Invitrogen) for 10 min and plated in 35 mm culture dishes at a cell density of ~100 cells/dish on the day before the experiments. Na⁺ channel currents were measured by using the whole-cell patch-clamp technique in the voltage-clamp configuration at room temperature. hiPSC-CMs were not selected by action potential morphology, but the differentiation technique used results in predominantly ventricular-like cells.² To measure Na⁺ channel currents, pipettes (3 to 4 MΩ) were filled with a pipette solution containing (in mmol/L): CsCl 80, cesium aspartate 80, EGTA 11, MgCl₂ 1, CaCl₂ 1, HEPES 10, and Na₂ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L): NaCl 20, NMDG 90, TEA-Cl 20, CsCl 5, CaCl₂ 2, MgCl₂ 1.2, HEPES 10, and glucose 5 (adjusted to pH 7.4
SUPPLEMENTAL MATERIAL

with CsOH). The holding potential was -100 mV. A voltage step protocol ranging from -80 to +70 mV with steps of 10 mV was applied to establish the presence of Na\(^+\) channel currents. The peak current density was used to plot current-voltage (I-V) curves. As before, nifedipine (10 \(\mu\)M, Sigma) was added in the bath solution to block L-type Ca\(^{2+}\) channel currents.\(^3\) Steady state activation and inactivation were characterized by Boltzmann functions. Macroscopic inactivation was fitted to a single exponential function.
SUPPLEMENTAL MATERIAL

Supplemental Results

Overexpression of full-length SCN5A has no effect on the expression of PERK expression

hiPSC-CMs were transfected with full-length SCN5A constructs at two different doses (2 μg and 3 μg). The expression of PERK was examined by Western blot. The results showed that the overexpressed full-length SCN5A had no effect on the expression of PERK (Fig. 1; P = 0.093).

Downregulation efficiency of PERK by pGIPZ lentiviral anti-PERK shRNAmir

hiPSC-CMs were divided into control (untreated) and experimental groups. Cells were pre-infected by pGIPZ lentiviral anti-PERK shRNAmir. The expression of PERK were examined at 48h by Western blot. The results showed the PERK knockdown efficiency was 60.0% (95% CI 55.3%, 64.7%; P = 0.024) at 48 h. A representative Western blot was shown in Fig. 2.

Membrane localization of full-length Na⁺ channels

To examine the cellular localization of full-length Na⁺ channels, a SCN5A construct tagged on the carboxyl terminal end with green fluorescent protein (SCN5A-GFP) was overexpressed in hiPSC-CMs. The detailed characteristics of this construct have been reported previously.¹ These characteristics include comparable gating and cellular distribution to that of wild-type channels. As has been previously reported, a portion of the full-length cardiac sodium channel colocalizes with the cell membrane (Fig. 3).

Sodium channel gating is unchanged by treatments

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Fig. 4 and Table 1 show that there were only minor changes in Na\textsuperscript{+} channel gating with AngII, PERK shRNA, or scrambled shRNA. These changes are not likely to be sufficient to explain the large effects of these treatments on peak Na\textsuperscript{+} current.
**Supplemental Table 1.** Steady state activation and inactivation parameters of sodium currents.

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$, mV</td>
<td>$p$</td>
</tr>
<tr>
<td>Control</td>
<td>-12.3 ± 0.5</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>Ang II-treated</td>
<td>-11.6 ± 0.6</td>
<td>0.47</td>
</tr>
<tr>
<td>Pre-infected by</td>
<td>-12.6 ± 1.0</td>
<td>0.47</td>
</tr>
<tr>
<td>PERK shRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-infected by</td>
<td>-12.3 ± 1.3</td>
<td>0.38</td>
</tr>
<tr>
<td>scrambled shRNA</td>
<td></td>
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</tbody>
</table>

$V_{1/2}$ is midpoint of the Boltzmann function. The value, $k$, is the slope factor of the Boltzmann function. n=5 for each group.
**Supplemental Figures**

**Supplemental Figure 1.** The effect of overexpression of full-length SCN5A on the PERK expression level. hiPSC-CMs were transfected with full-length SCN5A constructs at two different dose (2 μg and 3 μg). The expression of PERK were examined by Western blot at 48 h.
Supplemental Figure 2. The downregulation efficiency of PERK by pGIPZ lentiviral anti-PERK shRNAmir. Cells were pre-infected by pGIPZ lentiviral anti-PERK shRNAmir. The expression of PERK was examined at 48 h.
Supplemental Figure 3. The distribution of Na\(^+\) channels encoded by full-length SCN5A labeled with GFP. hiPSC-CMs were transfected with a GFP-labeled, full-length SCN5A construct. The full-length Na\(^+\) channel protein is shown in green. The cell membrane and nuclei are shown in red and blue, respectively. The co-localization of full-length Na\(^+\) channels is shown in the merged image and indicates Na\(^+\) channels at the membrane surface consistent with cellular electrophysiology.
Supplemental Figure 4. The $\text{Na}^+$ current macroscopic time constants of inactivation as a function of voltage for control iPS-hCMs and those treated with AngII, PERK shRNA, or scrambled shRNA ($P = 0.085$).
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References

