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Post-Transcriptional Alterations in the Expression of Cardiac Sodium Channel Subunits in Chronic Heart Failure

Stephen Zicha^{2,3}, Victor A. Maltsev⁴, Stanley Nattel^{2,3}, Hani N. Sabbah¹, and Albertas I. Undrovinas¹

¹*Division of Cardiovascular Medicine, Department of Medicine, Henry Ford Heart and Vascular Institute, Detroit, Michigan, USA*

²*Department of Medicine and Research Center, Montreal Heart Institute and University of Montreal, Montreal, Quebec, Canada*

³*Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada*

⁴*Gerontology Research Center, National Institutes of Health, Baltimore, Maryland, USA*

Abstract

Clinical and experimental evidence has recently accumulated about the importance of alterations of Na⁺ channel (NaCh) function and slow myocardial conduction for arrhythmias in infarcted and failing hearts (HF). The present study evaluated the molecular mechanisms of local alterations in the expression of NaCh subunits which underlie Na⁺ current (I_{Na}) density decrease in HF. HF was induced in 5 dogs by sequential coronary microembolization and developed approximately 3 months after the last embolization (left ventricle, LV, ejection fraction = 27±7%). 5 normal dogs served as a control group. Ventricular cardiomyocytes (VCs) were isolated enzymatically from LV mid-myocardium and I_{Na} was measured by whole-cell patch-clamp. The mRNA encoding the cardiac-specific sodium channel (NaCh) α-subunit Na_v1.5, and one of its auxiliary subunits β1 (NaChβ1), was analyzed by competitive RT-PCR. Protein levels of Na_v1.5, NaChβ1 and NaChβ2 were evaluated by Western blotting. The maximum density of I_{Na}/C_m was decreased in HF (n=5) compared to control hearts (32.3±2.6 vs. 50.8±6.5 pA/pF, mean±SEM, n=5, P<0.05). The steady-state inactivation and activation of I_{Na} remained unchanged in HF compared to control hearts. The levels of mRNA encoding Na_v1.5, and NaChβ1 were unaltered in failing hearts. However, Na_v1.5 protein expression was reduced about 30% in HF, while NaChβ1 and NaChβ2 protein were unchanged. We conclude that experimental HF in dogs results in post-transcriptional changes in cardiac NaCh α-subunit expression.

Keywords

heart failure; sodium channel; β-subunits; patch clamp; RT-PCR; Western blot

Introduction

Sudden cardiac death, due largely to ventricular tachyarrhythmias, remains a major public health care problem and accounts for nearly 40% of all deaths in patients with heart failure (HF). Local or regional electrical instabilities are considered a major risk factor in sudden cardiac deaths.¹ In addition to arrhythmias, another important potential consequence of slow

Address for correspondence: Albertas I. Undrovinas, PhD, Henry Ford Hospital, Cardiovascular Research, Education & Research Bldg. Room 4015, 2799 West Grand Boulevard, Detroit, MI 48202-2689, Phone: (313)-876-1321, Fax: (313)-876-3001, E-mail: AUNDROV1@HFHS.ORG.

trans-myocardial conduction is poor hemodynamic performance of the failing heart. In fact, patients with HF frequently manifest intra-ventricular conduction delays, which can be treated by cardiac resynchronization therapy.² Since the primary function of Na⁺ channels (NaChs) is to provide the electrical energy for cardiac impulse propagation, NaCh alterations may destabilize electrical function within diseased myocardium. Previous studies did not reveal changes in sodium current density (I_{Na}) in dogs with pacing-induced HF³ or in levels of mRNA encoding cardiac NaCh in patients with terminal HF.⁴ The recent discovery of transmural regional differences in NaCh expression^{5, 6} that have not been addressed in the previous studies^{3, 4} raises the question about the importance of local alterations of NaChs in HF. Indeed, down-regulation of NaChs has been found in canine chronic atrial fibrillation⁷ and chronic heart failure⁸ models and in myocytes from the epicardial border zone of infarcted hearts.⁹ Recent studies have indicated that alterations of NaChs, including 35% downregulation of I_{Na} , may contribute to the genesis of cardiac arrhythmias in a genetically engineered mouse HF model.¹⁰ Reduction in I_{Na} can: 1) slow ventricular conduction and cause repolarization abnormalities as in patients with Brugada syndrome¹¹ and ventricular arrhythmias, 2) slow myocardial conduction to cause sustained, isolated conduction defects¹² and 3) promote re-entrant arrhythmias.¹³ All these studies point to the importance of I_{Na} reduction for ventricular arrhythmias.

The present study was undertaken to evaluate the molecular mechanism of previously shown I_{Na} downregulation in chronic heart failure.⁸ By using a randomly chosen subset of animals reported in a previous study⁸, we investigated local alterations of expression of NaCh subunits in chronic HF.

The cardiac NaCh is comprised of the main pore forming α -subunit (Na_v1.5) as well as auxiliary β 1 (NaCh β 1), and β 2 subunits (NaCh β 2)¹⁴, which are transmembrane proteins that modulate α -subunit function. The auxiliary β 1-subunit has been implicated in regulation of Na_v1.5 kinetics associated with the LQT3 mutation¹⁵, aggravation of NaCh dysfunction in Brugada syndrome¹⁶, modification of block of NaCh by fatty acids¹⁷ and lidocaine¹⁸, and modulation of trafficking of Na_v1.5¹⁹. The physiological role of the β 2 subunit in heart is still under investigation, but it is known that to play a role in cell adhesion.^{14, 20} In brain, the β 2 subunit affects steady-state activation and inactivation of NaChs.²¹

To avoid known difficulties (interacting diseases, drug therapies, problems in sample procurement, etc) in human studies, a reproducible canine chronic HF model that mimics coronary artery disease and infarction was employed in the study. This HF model manifests extensive and sustained depression of ventricular function, marked ventricular ectopy, and sudden death in ~13% of animals.^{22, 23} Multiple diffuse infarctions were produced by sequential coronary embolizations with microspheres. Chronic HF develops approximately 3 months after the last embolization. To circumvent potential misinterpretations due to transmural regional differences in NaCh subunits expression⁵, voltage-clamp studies, competitive RT-PCR, and Western blots were performed using LV mid-myocardial slices and enzymatically-isolated cardiomyocytes (VCs) from these slices.

Materials and Methods

Canine Chronic Heart Failure Model

The dog model of chronic HF was previously described in detail²². Healthy mongrel dogs, weighing between 24 and 31 kg, underwent multiple sequential coronary microembolizations, to produce HF. Embolizations were performed 1 to 3 weeks apart and were discontinued when LV ejection fraction, determined angiographically, was $\leq 35\%$. At the time of harvesting the heart (~3 months after the last microembolization) LV ejection fraction was $27 \pm 7\%$. To test our hypothesis, 5 dogs with chronic HF and 5 weight-matched controls were randomly selected

for patch-clamp experiments and heart tissue samples were frozen for further molecular biology analysis. This study has been approved by the Henry Ford Health System Institutional Animal Care and Use Committee.

Cardiomyocyte Isolation

VCs were enzymatically isolated from slices of the apical third of the LV midmyocardium as previously described.²⁴ The yield of viable, Ca^{2+} -tolerant, rod-shape VCs isolated from both normal and failed hearts varied from 20% to 80%.

Patch-Clamp Measurements

I_{Na} was recorded with whole cell patch clamp as previously reported.²⁵ The pipette solution contained (in mM): CsCl 133, NaCl 5, ethylene glycol-bis (beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 10, MgATP 5, tetraethylammonium 20, HEPES 5 (pH 7.3 with CsOH). The patch pipette tip resistance was 0.5-0.8 M Ω . Whole cell currents were measured at room temperatures (22°-24°C) in symmetric Na^+ (5/5 mM, inside/outside) conditions, with a bath solution containing (in mM): NaCl 5, CsCl 133, MgCl_2 2, CaCl_2 1.8, nifedipine 0.002, HEPES 5 (pH 7.3 with CsOH). We used low symmetrical 5 mM / 5 mM Na^+ concentrations to reliably control the membrane potential during membrane current measurements. This is the only known way to measure a relatively large peak Na^+ current correctly using the standard patch clamp technique. This approach is generally accepted in the field; and has been applied widely to measure Na^+ current in canine⁹ and human²⁶ cardiomyocytes. To minimize spontaneous steady-state availability and activation shifts²⁵, the current measurements were performed approximately 20-25 min after the membrane rupture when any shifts have reached steady-state. Moreover, these shifts are likely to be species-dependent and are negligible in dog cardiomyocytes (our unpublished data) unlike in rat cardiomyocytes²⁵ or sheep Purkinje cells.²⁷ The peak of I_{Na} in these conditions ranged from 2 nA to 10 nA. The cell capacitance was measured by means of voltage ramp of 20 mV and 2 ms duration applied from -80 mV. The quality of the voltage clamp was controlled in each cardiomyocyte by estimating deviation (V_{dev}) from voltage command as previously described.²⁵ Satisfactory voltage control was assumed if $V_{\text{dev}} < 2$ mV, and only those cells which met this criterion were included in the study. For current density evaluation, raw currents were capacity and leak subtracted (examples of the raw recordings are provided in Fig 1 A,B).

RNA Isolation

Sections of mid-myocardial tissue were removed from the dogs and frozen immediately in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen) followed by chloroform extraction and isopropanol precipitation. Genomic DNA was eliminated by incubating in DNase I (0.1U/ μL , 37°C) for 30 minutes followed by acid phenolchloroform extraction. RNA was quantified by spectrophotometric absorbency at 260 nm, purity confirmed by A_{260}/A_{280} ratio and integrity evaluated by ethidium-bromide staining on a denaturing agarose gel. RNA samples were stored at -80°C in RNase-free Resuspension Solution (Ambion).

Primers

Primers specific for canine $\text{Na}_v1.5$ and $\text{Na}\beta1$ genes (Gene specific primers, GSPs) were designed based on previously published sequences (GenBank accession number AF017428). Chimeric primer pairs for the synthesis of the RNA mimic were constructed with a sequence homologous to rabbit cardiac α -actin flanked on both 5' and 3' sides by the GSPs. An 8-nucleotide sequence, GGCCGCGG, corresponding to the 3' end of the T7 promoter was conjugated to the 5' end of each forward chimeric primer.

Synthesis of RNA mimic

Reverse transcription was used to synthesize first-strand cDNA from dog ventricular total RNA sample. This cDNA was used as a template for the subsequent PCR steps. First, a PCR reaction was performed with the chimeric mimic primers. The product from this PCR consists of a 460-bp rabbit α -actin sequence, flanked at each end by either the dog Na_v1.5 or Na β 1 gene-specific sequence. In addition, there is an 8-bp sequence corresponding to the 3' end of the T7 promoter at the 5' end of the PCR product. A second PCR was performed with the T7 promoter as the sense primer, as well as the anti-sense GSP (either Na_v1.5 or Na β 1). The DNA product from this PCR contains the full T7 promoter and was gel-purified using the QIAquick Gel Extraction Kit (Qiagen Inc.). This DNA template was used for the subsequent *in vitro transcription* reactions using the T7 mMESSAGE MACHINE kit (Ambion Inc.) to create the RNA mimic. Any potential DNA contamination was removed after the reaction with RNase-free DNase I (30mins. at 37°C), followed by a phenol/chloroform extraction and isopropanol precipitation. The final RNA mimic contains the GSPs flanking a 460-bp rabbit α -actin sequence. The correct size was verified on a denaturing RNA gel. Pre-determined concentrations of RNA were run alongside the mimics in order to create a standard curve with which the concentration of the mimic was determined.

Reverse Transcription

Reaction mixtures containing serial 10-fold dilutions of the RNA mimic along with 1 μ g of total RNA sample were prepared and denatured at 65°C for 15 minutes. Reverse transcription was conducted in a 20- μ L reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 1 mM dNTPs (Roche), 3.2 μ g random primers p(dN)₆ (Roche), 5 mM DTT, 50 U RNase inhibitor (Promega), and 200 U M-MLV RT (Gibco RBL). The first strand synthesis was conducted at 42°C for 1 hour and then the enzymes were deactivated at 99°C for 5 minutes.

PCR

The reverse transcription products were used as a template for the subsequent PCR amplification. 25- μ L reaction mixtures were prepared containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 1 mM dNTPs, 0.5 μ M of each gene specific primer, 0.625 mM DMSO and 2.5 U of *Taq* Polymerase (Gibco BRL). The reactions were hot-started at 93°C for 3 minutes of denaturing before the *Taq* polymerase was added. The reactions were then cycled 30 times; with each cycle consisting of 30 seconds of denaturing at 93°C, 30 seconds of annealing at 55°C, and 30 seconds of elongation at 72°C, and finishing with a final elongation step of 5 minutes at 72°C.

Quantification of PCR Products

PCR products were visualized under UV light with ethidium bromide staining on a 1.5% agarose gel. The images were captured using a "Nighthawk" camera under UV light, and the band density was determined using Quantity One software (PDI). A low DNA mass ladder (Invitrogen) was used to determine the size of both the target and mimic bands, and to create a standard curve for each gel from which the band density could be quantified. The band density was converted to an absolute quantity using the standard curve and molecular weight correction for each band. The natural logarithm (LN) of the ratio of target over the mimic concentration should be linearly related to the logarithm of initial amount of mimic. Therefore, the x-intercept (LN target/mimic = 0) is the point where the mimic concentration and the target concentration are equal (ratio of target/mimic = 1). The LN of the target/mimic ratio was plotted against the LN of the mimic, and a linear regression gave the calculated x-intercept for each sample. The absolute amount of gene-specific mRNA was calculated from the x-intercept of each data set.

Western Blot Studies

Total protein was isolated from normal and CHF dog left ventricle samples that had previously been frozen. 1 g of tissue was homogenized in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1xPBS) and a protease inhibitor cocktail (10 mM β -mercaptoethanol, 10 μ g/mL PMSF, 5 μ g/mL aprotinin, 0.1 mg/mL benzamidine, 1 μ g/mL pepstatin A, 1 μ g/mL leupeptin, 100 mM sodium orthovanadate). All protease inhibitors were obtained from Sigma Chemicals and all procedures were performed on ice. The proteins were fractionated on 8% SDS-polyacrylamide gels and then transferred electrophoretically to Immobilon-P polyvinylidene fluoride membranes in 25 mM Trisbase, 192 mM glycine and 5% methanol. The membranes were blocked in 5% non-fat dry milk (NFDM, BioRad) in TTBS (Tris-HCl 50 mM, NaCl 500 mM; pH 7.5, 0.05% Tween-20) for 2 hours at room temperature and then probed with primary antibody at a dilution of 1:200 for 4 hours at room temperature in 5% NFDM TTBS. The anti-pan Na ν antibody was obtained from Alomone Labs (ASC-003) while the Na β 1 and Na β 2 antibodies were a kind gift from Dr. Isom.¹⁴ The membranes were subsequently washed three times in TTBS and then re-blocked in 5% NFDM in TTBS for 10 minutes and incubated along with a HRP-conjugated goat anti-rabbit secondary antibody (Jackson Immuno Labs) for 40 minutes in 5% NFDM in TTBS at a dilution of 1:10,000. The membranes were washed three more times in TTBS before antibody detection was performed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). The films were scanned with a PDI 420oe laser densitometer and the density of the bands was calculated using Quantity One software (PDI).

Experimental Protocols and Data Analysis

Data were acquired and analyzed using pClamp 8 software (Axon Instruments Inc.). The experimental protocols and data analysis were similar to those we previously reported.²⁵ The steady-state availability parameters ($V_{1/2A}$, the midpoint and k_A , the slope of the relationship) were measured by a standard double-pulse protocol with a 2 s-duration prepulse to a voltage (V_p) ranging from -140 mV to -40 mV. I_{Na} at each prepulse voltage was normalized to maximum I_{Na} measured at a prepulse voltage of -140 mV and fitted to a Boltzmann function $A(V_p)$:

$$A(V_p) = 1 / (1 + \exp((V_p - V_{1/2A})/k_A)) \quad (1)$$

The steady-state activation parameters were determined from the I_{Na} -voltage relationships by fitting data points of the normalized current with the function:

$$I_{Na}(V_t)/C_m = G_{max} * (V_r - V_t) / (1 + \exp((V_{1/2G} - V_t)/k_G)) \quad (2)$$

Where G_{max} is a normalized maximum Na $^+$ conductance, V_r is a reversal potential; $V_{1/2G}$, and k_G are the midpoint and the slope of the respective Boltzmann function underlying steady-state NaCh activation. The data points for I_{Na}/C_m were fitted by Clampfit 8 software using an optional 'Custom function' with four independent parameters corresponding to G_{max} , V_r , $V_{1/2G}$, and k_G . The peak current (I_{Na})-voltage relationship was determined in each cell with a series of depolarizing pulses of 50 ms duration applied to different testing voltages (V_t) at a rate of 0.5 Hz from a holding potential of -150 mV. I_{Na} density at each membrane potential was determined from these I_{Na} -voltage relations as the peak I_{Na} normalized to membrane electric capacitance, C_m , measured by a voltage ramp pulse with a slope (dV/dt) of -10 V/s from -80 mV to -100 mV. The C_m values were calculated according to $C_m = (I_{ramp} - I_{ss})/(dV/dt)$, where I_{ss} is steady-state level of membrane current at -100 mV and I_{ramp} is membrane current at the end of the ramp pulse.

Chemicals

All chemicals for which a source is not explicitly mentioned above were purchased from Sigma Chemical Co (St. Louis, MO).

Statistics

If not specifically stated, all measurements are reported as mean $\pm$ SEM with 'n' representing number of hearts. Comparison between mean values was performed with unpaired Student's t-test. A two-tailed probability <0.05 was taken to indicate statistical significance.

Results

I_{Na} density is significantly decreased in chronic HF

The whole-cell Na^+ currents were normalized to cell membrane capacitance, which on average was significantly higher in VCs isolated from failing hearts (FVC) compared to those isolated from normal hearts (NVC), 200 ± 12 pF (n=30) vs. 138 ± 10 pF (n=22, $P<0.05$), respectively. I_{Na} density in FVC was decreased compared to NVC over a wide range of membrane potentials. Figures 1A and B show raw I_{Na} recordings at different membrane potentials in NVC and FVC, accordingly. Figure C represents superimposed current voltage plots of average data obtained in 5 normal and 5 failing hearts. The maximum density of I_{Na} is significantly lower in failing hearts than in normal hearts (-33.2 ± 4.4 vs. 50.0 ± 4.9 pA/pF, mean $\pm$ SEM, $P<0.05$, Fig. 1D). The current was completely blocked by $25\mu M$ tetrodotoxin, a specific blocker of I_{Na} (not shown). The currents reversed at around 0 mV (see Fig 1C), as expected for the symmetrical Na^+ concentration conditions used in our experiments.

I_{Na} density changes were not related to steady state activation and availability

We measured steady-state activation and availability in each cell to rule out the possibility that the observed changes in I_{Na} density were due to alterations of these parameters. We found that voltage dependency of both steady-state activation (see Fig.1 theoretical curves) and availability (Fig.2) were unchanged by heart failure. Both NVCs and FVCs exhibited identical I_{Na} activation thresholds, over a range from -60 mV to -55 mV. For both cell types, the currents reached a maximum peak density at voltages close to -30 mV, similar to findings of others in normal canine cardiomyocytes under similar conditions.⁹

Western Blot Studies

Figure 3A shows results from a Western blot probed with an anti-pan Na_v antibody. A single band at ~ 220 kDa was detected. Specificity of the band was confirmed by preincubation of the antibody with a control antigen against which the antibody was raised (last two lanes). Normal dogs were found to have an average band density of 13.4 ± 1.9 OD units, compared to an average density of 10.5 ± 1.9 OD units in HF dogs (n=5 per group, $p<0.05$, Figure 3B).). Figure 3C shows results from Western blots probed with an anti- $NaCh\beta 1$ antibody. Two bands were detected, one at 43 kDa, which corresponds to the anticipated fully glycosylated protein, and a smaller faint band at 36 kDa, that corresponds to an incompletely glycosylated product. Figure 3D shows that the relative amount of protein corresponding to the 43 kDa band was unchanged in failing hearts (4.8 ± 0.8 normal hearts vs. 3.7 ± 1.0 HF, $p>0.05$, n=5/group). Fig. 3E shows results of Western blots probed with an anti- $NaCh\beta 2$ antibody. A single specific band at 35kDa was detected in both normal and failing dog samples. . There was no difference in the amount of $NaCh\beta 2$ protein in failing hearts compared to normal hearts (4.8 ± 0.1 NH vs. 4.1 ± 0.4 HF arbitrary OD Units, n=5/group, $p=NS$, Fig 3F).

Competitive RT-PCR of NaCh subunits

Figure 4 shows examples of Na_v1.5 competitive RT-PCR using total RNA isolated from normal heart (NH, Fig. 4A) and HF (Fig. 4B) mid-myocardial slices. In both cases, lane 0 contains 100 ng of DNA Mass Ladder used to create a standard curve for each gel. Lanes 1 through 5 were obtained with serial dilutions of the RNA mimic along with 1 µg total RNA. The upper bands represent the internal standard PCR product, while the lower bands are the target Na_v1.5 bands co-amplified with the mimics in the same reaction tube. As the mimic concentration decreases from left to right, the target band gets stronger, demonstrating the competition between mimic and target. For each experiment, the sample Na_v1.5 mRNA concentration was calculated based on the target and mimic band intensities at the following dilutions: 7 ng, 700 pg, 70 pg, 7 pg and 0.7 pg. Figure 4C shows the means for each dilution of mimic used for the quantitative analysis. Figure 4D shows the average absolute concentration of mRNA. The concentrations of Na_v1.5 mRNA in normal and HF samples were 389±99 and 396±109 attomol/µg total RNA respectively (n=4/group, P=NS).

A similar analysis was performed for the auxiliary β1 subunit. Figures 5A and 5B show representative gels from NaChβ1 competitive RT-PCR experiments for normal and failing hearts, respectively. Total RNA from mid-myocardial regions of normal and CHF dogs along with the following dilutions of mimic were used: 537 pg, 53.7 pg, 5.4 pg, 537 fg and 53.7 fg. The means for each dilution used for the quantification are shown in Fig 5C, while the bar chart in Figure 5D shows the average absolute amount of mRNA encoding the NaChβ1 subunit: 72 ±23 attomol/µg total RNA for normal dogs and 74±20 attomol/µg total RNA for CHF dogs.

Discussion

We have demonstrated that NaCh function and protein expression are downregulated in a canine model of ischemic cardiomyopathy. Expression of mRNAs encoding Na_v1.5, and the NaChβ1 auxiliary subunit remained unchanged in failing hearts. The protein levels for both the β1 and the β2 subunits were unchanged. Our results suggest a post-transcriptional mechanism for Na_v1.5 subunit expression changes in failing hearts.

Molecular studies support electrophysiological observations of decreased I_{Na} in heart failure

Peak I_{Na} density was significantly reduced in the mid-myocardial region of dogs with chronic heart failure. We searched for a molecular mechanism that could underlie these changes. We found no change in the absolute amount of mRNA encoding Na_v1.5 and NaChβ1 subunits in HF. Western blotting revealed a significant difference in the amount of Na_v1.5, but not NaChβ1 and NaChβ2, subunit protein. This would suggest that the changes seen at the physiological level are due to post-transcriptional modifications in the processing of Na_v1.5.

Possible clinical relevance

The I_{Na} downregulation found in the present study may provide a candidate mechanism potentially contributing to the development of ventricular arrhythmias in HF related to coronary artery disease. Na⁺ and K⁺ current ⁴ changes can both contribute to arrhythmogenesis in HF. Indeed, together with geometric non-uniformities (structural alterations) within the healed infarction tissue (including fibrosis or myocardial scarring) and decreased cell-to-cell coupling in HF ²⁸, the decline of functional NaCh density ⁸ reported in the present study can contribute to unidirectional block, slowed impulse propagation, and re-entrant arrhythmias in the failing heart. It has recently been shown that similar changes in I_{Na} can slow myocardial conduction and cause sustained, isolated conduction defects. ¹²

Second, this mechanism can contribute to the pro-arrhythmic effect of Class I antiarrhythmic drugs, ²⁹ as further blockade of down-regulated NaCh can make impulse propagation even

worse. Class I antiarrhythmics agents have not been studied in randomized heart failure trials, but were associated with increased mortality in studies of patients at high risk for ventricular arrhythmia, including patients with left ventricular dysfunction.³⁰⁻³² Because this increase in mortality is thought to be due to pro-arrhythmic properties of the drugs, further trials in heart failure patients are unlikely to occur.³²

Possible mechanisms of I_{Na} post-transcriptional downregulation

The abundance of NaChs in the cardiomyocyte surface membrane is dependent on the balance between NaCh protein insertion and breakdown/internalization. Recent studies highlighted the importance of signal transduction in modulation of cell surface NaCh expression through the PKA, G protein, PKC and calpain pathways. (19, 33, 34, for review see³⁵) Activation of PKA or G protein stimulatory subunits *via* β -adrenoreceptors increases abundance of NaChs by promoting channel insertion into cardiomyocyte membranes.^{19, 33, 34} NaCh insertion by this mechanism may be impaired in failing hearts because of down-regulated β -adrenoreceptors.^{36, 37} On the other hand, activation of Ca^{2+} -dependent PKC and calpain by augmented $[Ca^{2+}]_i$ reduces surface expression of NaChs by accelerated NaCh internalization in adrenal chromaffin cells^{38, 39}. Defective Ca^{2+} handling resulting in elevation of diastolic $[Ca^{2+}]_i$ and delayed Ca^{2+} uptake by the SR,^{40, 41} together with increased activity of Ca^{2+} -dependent PKC in HF,^{42, 43} may also accelerate NaCh internalization, resulting in the reduced I_{Na} density reported here. Thus, these Ca^{2+} -mediated intracellular signaling pathways may be important for post-transcriptional abnormalities of NaCh processing in HF. In support of this idea we have recently demonstrated that buffering of $[Ca^{2+}]_i$ by 24-hour exposure of cardiomyocytes from failing dog hearts to cell permeable BAPTA-AM (20 mM) may partially restore NaCh density.⁸ Cytoskeleton damage documented in HF⁴⁴ may also contribute to reduced I_{Na} .^{25, 45} Analysis of the contribution of these mechanisms to NaCh regulation in HF awaits further studies.

Study limitations

To avoid contamination by cells from different myocardial locations, we studied changes in NaChs expression in LV mid-myocardium from the apex region. Accordingly, the difference between our results and the previous finding of unchanged I_{Na} density in total LV of dogs with pacing-induced HF⁴⁶ can be explained by the HF model (pacing vs. multiple infarctions), as well as potentially by local variations in NaCh density. A more detailed understanding of differences in NaCh expression changes across the ventricular wall awaits further exploration.

The antibody used to determine $Na_v1.5$ protein expression was not specific for this subunit, but rather detects the presence of all Na_v isoforms. An unexpected role for the brain-type NaCh in heart has been recently discovered.^{47, 48} This study showed that $Na_v1.1$, $Na_v1.3$, $Na_v1.5$ and $Na_v1.6$ are differentially located within the VCs of mice and that the major cardiac isoform, α -subunit $Na_v1.5$, is present in the intercalated disks.⁴⁷ The highly TTX-sensitive brain isoforms, $Na_v1.1$, $Na_v1.3$, and $Na_v1.6$, are located in the transverse tubules and can generate a very small current detectable only after activation with β -scorpion toxin.^{47, 48} However, it has been shown previously that $Na_v1.5$ is the predominant isoform responsible for cardiac I_{Na} , so the results presented here most likely reflect the expression of this principal subunit. Recently additional $\beta 3$ and $\beta 4$ subunits were discovered in brain and heart.^{21, 48-50} In ventricular myocytes, β subunits manifest distinct sub-cellular localization.⁴⁸ The discovery of additional β -subunits increases the potential complexity of NaCh structure and raises further questions about the role of β subunits in heart. Unfortunately, no control peptide was available for negative control studies using the NaCh $\beta 1$ and NaCh $\beta 2$ antibodies. Also, contamination from the nerves could interfere with PCR and Western blot studies and mask changes in β subunits expression. Accordingly, use of cardiomyocyte suspension furthering future studies may unmask possible changes of β -subunits expression in HF if such exist. Nevertheless, the

data shown here are in accordance with other previously published results using the same antibody.¹⁴

In conclusion, experimental chronic HF results in down-regulation of I_{Na} in LV *via* post-transcriptional mechanisms.

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Abbreviations and Units

HF, heart failure
 I_{Na} , inward sodium current, nA
 I_{Na}/C_m , sodium current density in pA/pF
 LV, left ventricle
 Na_v , voltage-gated sodium channels
 $Na_v1.5$, main α subunit of the cardiac sodium channel isoform
 NaCh, sodium channel
 $NaCh\beta1$, $NaCh\beta2$, sodium channel auxiliary β -subunits
 OD, optical density
 RT, reverse transcription
 PCR, polymerase chain reaction
 TTX, tetrodotoxin, a specific inhibitor of sodium channels
 VCs, ventricular cardiomyocytes
 NVCs, FVCs, ventricular cardiomyocytes of normal and failing heart, respectively

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Figure 1.

Sodium current, I_{Na} , in ventricular cardiomyocytes of dogs with chronic HF is downregulated. Representative examples of whole-cell I_{Na} current traces recorded at different membrane potentials in cardiomyocytes from normal (A) and failing (B) heart. Inset shows a schematic representation of the voltage clamp protocol; the testing voltage, V_t , increment was 5 mV. Amplitude and time calibration is the same for both experiments. C, average current-voltage relationships for peak Na^+ current density in normal (closed circles, n=5 dogs, 23 cardiomyocytes) and failing myocardium (open circles, n=5 dogs, 30 cardiomyocytes), respectively (* $P < 0.05$) analyzed for V_t values separated by 10 mV. Shown are mean $\pm$ SEM for n, number of hearts used in the study. The solid lines show theoretical curves fitted to data

points in accordance with Equation 2 (see methods). While maximum Na^+ conductance, G_{max} significantly decreased from 1.7 nS/pF to 1.2 nS/pF, other fit parameters remained almost unchanged in failing hearts. Particularly V_r , $V_{1/2G}$, and k_G were as follows (in mV): 3, -43, 6.1 vs. 2.1, -40, 5.3, normal vs. heart failure, respectively.

Figure 2.

Voltage dependency of the steady-state inactivation remains unchanged in failing hearts. A,B left panels - raw current traces recorded in normal (A) and failing (B) hearts in response to a voltage-clamp protocol (shown in inset) to evaluate steady-state inactivation, respectively. Panel C show average data on steady-state inactivation vs. membrane potential. Shown are mean $\pm$ SEM for n, number of hearts used in the study. Solid lines ($A(V_p)$) represent theoretical fit to Boltzmann function (Equation 1, Methods). Note, that mid-potential and slope for steady-state inactivation were similar in cardiomyocytes of normal and failing hearts, (-83.8 mV vs. -83.0 mV and 6.3 mV vs. 6.6 mV, respectively).

Figure 3.

Western blot analysis of cardiac Na⁺ channel Na_v1.5 subunit and its auxiliary subunits in normal and failing hearts. **A**, Result of Western blot using membrane protein fractions probed with anti-pan Na_v antibody. An anticipated single band corresponding to Na_v1.5 at 220 kDa is detected. The band disappears when primary antibody is pre-incubated with control antigen (last two lanes), indicating specificity of the band. **B**, Bar chart comparing the mean band intensities of Na_v1.5 protein between normal and heart failure dog mid-myocardial samples. Heart failure dogs had a significantly lesser amount of Na_v1.5 protein as compared to normal dogs: 10.5 ± 1.9 vs. 13.4 ± 1.9 arbitrary OD units respectively (n=5 for both groups, p<0.05). **C**, Example of a membrane probed with anti-NaChβ1 antibody. The 43 kDa band that corresponds to fully glycosylated NaChβ1 protein was identified in both samples on normal and failing hearts. The faint 36 kDa band may represent an incompletely glycosylated protein, and was not quantified. **D**, Bar chart comparing densities of NaChβ1 protein bands. No significant difference was found in the density of the 43 kDa band in failing hearts (3.7 ± 1.0) compared with normal hearts (4.8 ± 0.8, p>0.05, n=5/group). **E**, Shows an example of a Western blot probed with an NaChβ2 antibody. A single band was detected at 35 kDa. **F**, Mean data comparing the density of NaChβ2 in normal dogs and dogs with and heart failure. No significant difference was detected (4.8 ± 0.1 NH vs. 4.1 ± 0.4 HF arbitrary OD Units, n=5/group, p>0.05).

Figure 4.

Quantification of mRNA encoding $\text{Nav}1.5$ in normal and failing dog hearts by competitive RT-PCR. **A**, Shown is a representative gel of $\text{Nav}1.5$ competitive RT-PCR using total RNA isolated from mid-myocardial slices of normal dog heart. Lane 0 contains 100ng of Low DNA Mass Ladder used to make standard curve for each gel. Dilutions of mimic in lanes 1 through 5 are 7 ng, 700 pg, 70 pg, 7 pg and 0.7 pg. **B**, A sample of a $\text{Nav}1.5$ competitive RT-PCR result using heart failure total RNA from mid-myocardial sections. Dilutions are the same as in **A**. **C**, abundance of $\text{Nav}1.5$ was determined from a linear regression to the logarithmic ratio of optical density of amplified target DNA/mimic DNA versus logarithm of mimic concentration plot. Point of equivalence found, as regression line intercept with zero line, indicates the absolute target mRNA concentration. **D**, Histogram comparing the absolute concentrations of $\text{Nav}1.5$ mRNA evaluated by the graphic method shown in **C** in normal and heart failure mid-myocardium. There was no significant difference in the amount of $\text{Nav}1.5$ mRNA between normal and heart failure groups.

Figure 5.

Results of competitive RT-PCR for sodium channel $\beta 1$ subunit (NaCh $\beta 1$). **A**, An example of NaCh $\beta 1$ competitive RT-PCR using total RNA from normal dog midmyocardium. Lanes 1 through 5 contain 537 pg, 53.7 pg, 5.4 pg, 537 fg and 53.7 fg of mimic. **B**, shows an example of NaCh $\beta 1$ competitive RT-PCR using heart failure total RNA samples. The dilutions used are the same as in A. **C**, abundance of mRNA encoding NaCh $\beta 1$ was determined from a linear regression to the logarithmic ratio of optical density of amplified target DNA/mimic DNA versus logarithm of mimic concentration plot. Point of equivalence found, as regression line intercept with dashed zero line, indicate the absolute target mRNA concentration. **D**, Histogram comparing absolute amounts of NaCh $\beta 1$ mRNA in normal and heart failure canine midmyocardium. Normal dogs had 72.2 ± 23.3 attomol/ μ g total RNA compared to 74.3 ± 19.9 attomol/ μ g total RNA for heart failure dogs. This difference was not significantly different ($n=5$ for both groups, $p>0.05$).