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Myocardial Function with Reduced Expression of the Sodium-Calcium Exchanger

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Abstract

Background—The complete removal of the cardiac sodium-calcium exchanger (NCX1) is associated with embryonic lethality while its overexpression is linked to heart failure. To determine whether or not a reduced expression of NCX1 is compatible with normal heart structure and function, we studied two knockout mouse models with reduced levels of NCX1: a heterozygous global knockout (HG-KO) with a 50% level of NCX1 expression in all myocytes, and a ventricular specific KO (V-KO) with NCX1 expression in only 10-20% of the myocytes.

Methods and Results—Both groups of mice were evaluated at baseline, after trans-aortic constriction (TAC), and after acute or chronic beta-adrenergic stimulation. At baseline, the HG-KO mice had smaller hearts and the V-KO mice had larger hearts than their wild-type (WT) controls ($P < 0.05$). The HG-KO and their control WT mice had normal responses to TAC and beta-adrenergic stimulation. However, the V-KO group was intolerant to TAC and had a significantly ($P < 0.05$) blunted response to beta-adrenergic stimulation as compared to the HG-KO mice and WT controls. Unlike the HG-KO mice, the V-KO mice did not tolerate chronic isoproterenol infusion. Telemetric analysis of the ECG, body temperature and activity revealed a normal diurnal rhythm in all groups of mice, but confirmed shorter QT intervals along with increased arrhythmias and reduced R/P amplitude ratios in the V-KO mice.

Conclusions—Though NCX1 can be reduced by ½ in all myocytes without significant functional alterations, it must be expressed in more than 20% of the myocytes to prevent severe remodeling and heart failure in mouse heart.

Keywords

Heart Failure; Hypertrophy; Ventricular Dysfunction; Knock-out Mice

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Introduction

The cardiac sodium-calcium exchanger (NCX1) is a sarcolemmal protein that maintains Ca^{2+} homeostasis by exchanging 3 Na^+ ions for 1 Ca^{2+} ion.¹ Ca^{2+} enters the myocyte via L-type Ca^{2+} channels and triggers contraction by the additional Ca^{2+} release from the sarcoplasmic reticulum (SR). Relaxation of myocytes is modulated by Ca^{2+} extrusion by NCX1 and resequestration by the SR Ca^{2+} ATPase (SERCA2). This NCX1 forward mode exchange is the primary mechanism of Ca^{2+} extrusion from the cell. In addition, the exchanger may transport Ca^{2+} back into the myocyte (reverse mode exchange) during heart failure or myocardial development.^{2,3} Many Ca^{2+} handling protein alterations are present in myocardial dysfunction and heart failure.⁴⁻⁶ At the molecular level, NCX1 is upregulated and SERCA2 is downregulated during chronic pressure overload and in mammalian models of heart failure.⁷ Thus, the expression level and activity of NCX1 in the heart is an important factor in understanding cardiac pathophysiology.

In a previous study from our group, mice with increased exchanger activity manifested compensated cardiac hypertrophy at baseline and a blunted response to β -adrenergic stimulation proportional to the amount of the exchanger overexpression.⁸ Moreover mice stressed by pregnancy, exercise or surgically induced pressure overload showed an accelerated maladaptive hypertrophy and transition to heart failure related to their NCX1 expression level. On the other hand, the complete removal of the exchanger from all tissues is associated with embryonic lethality in mice by day 11.⁹⁻¹¹ However, heterozygous global knock-out (HG-KO) mice, with about a 50% NCX1 expression level in all myocytes, survive.

Our group has also generated a ventricular specific NCX1 KO mouse model (V-KO) via the deletion of exon 11 using the Cre-Lox system directed by the MLC2v promoter.¹² In this model, NCX1 is detectable in only 10-20% of the ventricular myocytes. These V-KO mice surprisingly live to adulthood with reduced, but adequate function as assessed by echocardiography at 7.5 weeks of age. No myocardial biochemical adaptations to the absence of the exchanger were detected either by immunoblot for specific E-C coupling proteins or by transcription wide microarray analysis.

The expression level of the exchanger is important for proper myocardial function. In pathological conditions that manifest alterations in Ca^{2+} homeostasis, systolic and diastolic dysfunction is a common finding.⁶ Since overexpression of NCX1 leads to ventricular dysfunction, hypertrophy and failure,⁸ a reduction in exchanger expression and activity could be beneficial in minimizing maladaptive myocardial remodeling. On the other hand, a reduction in exchanger expression might be as deleterious as overexpression due to the perturbation of calcium homeostasis leading to Ca^{2+} overload. Previous studies with a heterozygous global KO mouse model from Takimoto's lab¹³ and our V-KO mouse model,^{12,14-16} did not fully address the effect of NCX1 knockdown in older mice or under stressed conditions. Therefore, we further evaluated our HG-KO and V-KO models to characterize their cardiac remodeling and function at baseline, and following hemodynamic stress from pressure overload or β -adrenergic stimulation in order to investigate whether or not a reduction in NCX1 expression could be beneficial, neutral or detrimental to myocardial function.

Methods

Generation of the KO Mice

Two lines of NCX1 knockout mice have been generated in our laboratory. First, exon 2 of NCX1 was ablated to create animals with non-functional exchanger in all cell types as previously described.¹¹ The heterozygous HG-KO mice have 50% exchanger activity in the

myocytes as determined by immunoreactivity assays.¹¹ Second, we developed a ventricular specific knockout mouse model (V-KO) via the Cre/loxP system as previously detailed.¹² Mutant mice were created in which exon 11 of the NCX1 gene was conditionally removed from the ventricular myocytes using the myosin light chain 2 promoter to activate Cre expression. This promoter is transcriptionally active at about embryonic day 8 through adulthood. These mice express NCX1 in only about 10-20% of the ventricular myocytes. The balance of the ventricular myocytes have no exchanger activity.¹² Control mice for the current study were littermates of either the HG-KO mice that were homozygous for the wildtype NCX1 allele or littermates of the V-KO mice that did not express cre, only flox. For simplicity, we refer to these mice as WT. The mice for both models are on a black Swiss background.¹⁴ A balance of mixed gender mice were used for all the studies at similar ages within a given study; no significant gender differences were noted in the evaluations for either model. All animal protocols, experiments and housing were approved by the UCLA institutional review committee.

Echocardiography

Echocardiography was performed on all the mice with a Siemens Acuson Sequoia C256 instrument (Siemens Medical Solutions, Mountain View, California) as previously described.^{8,17} The mice were sedated with either isoflurane vaporized in oxygen (Summit Anesthesia Solutions, Bend Oregon) or with Avertin (2,2,2 tribromo-ethanol 2.5% solution, .0016ml/gm body mass, Aldrich Chemical Corp, St. Louis MO). Left ventricular dimensions (EDD, end diastolic dimension; ESD, end systolic dimension; PWT, posterior wall thickness; VST, ventricular septal thickness) obtained from 2D guided M-mode were analyzed with AccessPoint software (Freeland System LLC, Santa Fe, New Mexico) during diastole and systole. Left ventricular mass was calculated as described by Tanaka et al.¹⁸ Heart rate, aortic ejection time and mitral inflow E & A wave amplitudes were determined from Doppler flow images. Indices of contractility such as left ventricular fractional shortening (LVFS), velocity of circumferential fiber shortening (VCF) and ejection fraction (EF) was obtained from the images. During the procedure, heart rates were maintained at physiological levels by monitoring their electrocardiograms (ECG) as previously described.¹⁷

Hemodynamic Assessment

Hemodynamics were assessed similarly to those previously described.⁸ Prior to assessment, all mice were anesthetized with a mixture of Ketamine (100 mg/kg), Xylazine (2 mg/kg), and Buprenorphine (0.1 mg/kg), intubated, and ventilated. A Millar 1.4 Fr catheter (Millar Instruments, Houston, Texas) was inserted via right carotid and placed into the left ventricle. The ECG, LV pressures and $\pm$ dP/dT data were recorded at baseline and after sequential 1-30 ng/Kg dose (0.1ml bolus intravenously) infusion of dobutamine given at 10-15 minutes intervals. Isoproterenol (ISO) (50 -1000 pg/Kg) was given only to the conditional ventricular KO mice and their control group in a similar manner as with the dobutamine infusion experiments. All the data were acquired, digitized and analyzed with HEM software (Notocord Systems, Croissy sur Seine, France).

Trans-aortic Constriction

Pressure overload was induced by trans-aortic constriction (TAC) surgery as previously described.^{8,19} The aortic arch was exposed and constricted to the diameter of a 27 gauge needle in the anesthetized and ventilated mice. A subset of mice underwent sham surgeries identical to those described above, but without the aortic constriction. Mice were kept for 21 days post-TAC or sham. Their ventricular parameters were assessed by echocardiography prior to and 21 days post procedure. Pressure gradients across the constriction were measured in all survivor TAC mice at 21 days, by femoral artery catheterizations with a flame-stretched PE-50 catheter

and right carotid catheterizations with a Millar catheter as described above. Data from mice with gradients lower than 40 mmHg were excluded. After gradient confirmation, the Millar catheter was advanced into the LV chamber to measure LV pressures and $\pm$ dP/dT post-TAC.

Chronic Isoproterenol Infusion

HG-KO, V-KO and their wild-type littermate mice were anesthetized with isoflurane and miniosmotic pumps (Alzet model 2001, Durect Corp., Cupertino CA) were positioned subcutaneously. The pumps were filled with sterile saline (sham) or with isoproterenol to challenge the myocardium with different dosages (0.2, 0.4, or 0.8 mg/day) dispensed over 7 days. The sham and isoproterenol treated mice were echoed prior to pump implantation and at the end of the study to identify any ventricular remodeling and functional changes from baseline.

Morphologic Assessment

Morphologic assessment was performed at the end of the study in all mice. The heart and body weights along with their tibia lengths were obtained. Some hearts were stored in formalin for histology. Tissues were processed routinely, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Masson's trichrome stain. Tissue sections were examined and graded (0 to 3) for the presence of fibrosis and necrosis. Fibrosis was graded as follows: 1 = foci of interstitial fibrosis affecting less than 10% of the myocardium; 2 = foci of interstitial fibrosis affecting more than 10% of the myocardium, and 3 = replacement fibrosis. Necrosis was graded as follows: grade 1 = focal necrosis affecting less than 10% of the myocardium; grade 2 = multifocal necrosis affecting 10 to 30% of the myocardium, and grade 3 = diffuse necrosis affecting more than 30% of the myocardium.

Telemetry

ECG radio telemetry transmitters (model #TA10ETA-F20, Data Sciences International, St. Paul, MN) were implanted in KO and WT mice as previously described.^{16,20,21} The mice were anesthetized and the transmitters implanted in the peritoneal cavity. After recovery, the unfiltered ECG waveforms, body temperatures and cage activities were collected for 30 seconds at 10 minute intervals for 1-2 months after implantation. Heart rates along with ECG amplitudes and waveform intervals were determined with the DSI analysis software (ART V4.0 and Physiostat V4.01). The data were separated by time of day for the differential analysis of light (inactive) and dark (active) cycle diurnal periods as presented in Table 2. Waveform data were visually scanned for waveform shape abnormalities and arrhythmias.

Statistical Methods

The data are shown as means and $\pm$ standard errors. Data were evaluated using 2 tailed *t* tests or analysis of variance (ANOVA) with Instat V3.05 statistical program (Graph Pad Inc, San Diego, California). In all statistical tests *P* < .05 was taken as significance.

Results

Baseline Morphology and Function

Figures 1 and 2, along with Table 1 present the baseline morphology and functional data for both mouse models. At baseline, there was no necrosis in any of the hearts. Further, there was no fibrosis in the WT and HG-KO mice (Fig 1A, 1C), while the V-KO mice already exhibited grade 1 focal interstitial fibrosis in the LV free wall by 12 weeks of age (Fig 1E - inset). The baseline echo and weight data in Table 1 were obtained from 10.5 to 11.5 week old mice just prior to TAC surgery. These data show that the WT mice from both groups are normal and not significantly different from each other. The mean baseline body weights of all groups of mice

were in the 24-28 gram range with no significant gender differences. However, the HG-KO and V-KO mice demonstrate some interesting differences from their WT littermates at baseline. Despite having the similar body weights, the HG-KO mice have significantly smaller hearts while the V-KO mice have larger hearts at this age as indicated by wall thickness and left ventricular mass estimates (Table 1 & Figures 2A & 2B, baseline values). This was confirmed by post mortem evaluation of the heart weight to tibia length ratio in a different set of WT and KO mouse hearts at 13 ± 1 weeks of age (Fig 2C, baseline values). The echo measurements also demonstrate that the HG-KO mice had normal chamber dimensions and function at baseline (Table 1 & Figure 2D, baseline values). However, the V-KO mice had a significant increase in ESD as reflected in reductions in functional parameters (LVFS, VCF & EF) at this age (Table 1 & Figure 2D, baseline values).

The mean age of death in the V-KO mice is about 14 weeks of age¹² with the oldest mouse surviving to 22 weeks. Thus, additional baseline echocardiography was performed at 14.2 ± 0.6 weeks of age on a separate group of 11 V-KO mice to document any further changes in morphology and function. When compared to the V-KO values in Table 1, the 3½ additional weeks of aging revealed: (1) a 46% increase in LV mass to 123 mg; (2) a 30% increase in posterior wall thickness to 0.85 mm; and (3) an additional 12% decline in ejection fraction to 47.1%. The echo data from the 4 sham V-KO mice at the same age shown in Figure 2 are consistent with the above observations. Thus after 3 months of age, the V-KO mice exhibit hypertrophic remodeling and dysfunction with progressive decompensation to heart failure and death by 22 weeks of age or earlier. All baseline echo parameters are normal in the HG-KO mice up to 18 months of age.

Response to TAC Induced Pressure Overload

After baseline echos, WT and KO mice from both genotypes underwent TAC surgery to increase their hemodynamic load; a sub-set of mice underwent sham surgery as a control. After 21 days, the sham and TAC mice were echoed again to reveal any remodeling or change in function. Following the echo, their pressure gradients were assessed, contractility and relaxation calculated from their $\pm dP/dT$ values and the morphometric measurements obtained at the end of the experiment.

The TAC and sham procedures were tolerated well in the HG-KO mice and their WT littermates with over a 90% survival rate at 21 days. The sham control HG-KO & WT mouse data are indistinguishable from their baseline values (Figs 2 A-C, baseline vs. sham) despite being 3 weeks older. The HG-KO & WT mice exhibited significant ($P < 0.05$) post-TAC hypertrophy with increased wall thicknesses (Figs 2A, 2B & 2C, TAC values). The EF was not affected by either sham or TAC surgery in HG-KO mice (Fig. 2D, sham & TAC values). Similarly, the peak contractility and relaxation values ($\pm dP/dT$) measured post-TAC were no different than baseline in these mice. However, the HG-KO mice exhibited modest LV diastolic dysfunction in their post-TAC $\pm dP/dT$ traces as previously reported by Takimoto et al¹³ in a similar mouse model.

On the other hand, the V-KO mice were intolerant to TAC with almost 80% mortality within the first week and no survivors past 21 days. Thus no V-KO TAC data are shown in figures 2 A, B & D. The V-KO HW/TL ratio data in figure 2C are from the two mice that died just prior to their scheduled 21 day echo; they are included only as a reference and not included in the statistical analyses. Unlike the TAC results, all but one of the V-KO mice survived the sham control surgery. As with the HG-KO mice, the sham morphometry and function data from wildtype littermates of the V-KO mice are indistinguishable from the non-sham mice (Fig. 2, WT baseline vs. sham). The sham V-KO mice are 3 weeks older than the baseline controls and exhibit significantly increased hypertrophy and dysfunction as described in the previous section (Fig. 2, V-KO sham).

Response to β -Adrenergic Stimulation

Both acute and chronic responses to beta agonists were evaluated (Figs 3 & 4). The contractility and relaxation were reduced at baseline in the V-KO mice with respect to the HG-KO and both groups of wild-type mice (Figs 3A & 3B). The response of V-KO mice to acute dobutamine infusions were significantly blunted ($P < 0.05$) at each dosage. The response to dobutamine was preserved in the HG-KO mice, as it was in the control group. All groups exhibited a similar magnitude of increase in heart rate in response to dobutamine. Isoproterenol (ISO), a non-specific β -adrenergic agonist, was only given to the V-KO mice to further evaluate their response. Both contractility and relaxation rates were significantly blunted as with the dobutamine challenge (Fig. 3C & 3D).

Figure 4A illustrates an example of ventricular fibrillation that commonly occurred in older V-KO mice (> 12 weeks of age) at baseline and during the course of the acute dobutamine infusion studies. In addition to ventricular fibrillation, we also observed premature ventricular contractions (PVC) and bigemini even at the lowest dosages in many mice. Figure 4B illustrates an entire dobutamine infusion study from a V-KO mouse. The LV pressure and contractility response at 3 and 6 ng of dobutamine show severe blunting. At the higher 15 and 30 ng doses the pressure and dP/dT values were depressed below the baseline at the time of peak heart rates (Fig 4B, arrows). This depression of pressures and contractility was observed in most other KO mice older than 12 weeks of age. All WT mice showed the normal response to dobutamine of increased pressures, dP/dt values and heart rates (Fig 4C).

Since the acute dobutamine and ISO infusions failed to rescue the V-KO myocardial function, we implanted osmotic pumps to evaluate the response to a chronic infusion to ISO and to saline (sham) in both mouse models. Baseline echo data taken prior to implantation were consistent with those from the pre-TAC mice detailed in table 1. HG-KO mice and their wildtype littermates ($n = 4$ HG-KO & 4 WT; age 11.1 weeks) were implanted for 7 days receiving 0.8mg per day of ISO; similarly implanted sham controls ($n = 2$ HG-KO & 2 WT; age 11.8 weeks) received saline for 7 days. After 7 days treatment, both WT and HG-KO mice receiving ISO exhibited hypertrophy with significant increases in VST, PWT & LVM ($P < 0.05$) while retaining normal ventricular function (LVFS, VCF & EF) compared to baseline. The hypertrophic response was confirmed by significant ($P < 0.05$) increases in the HW/TL ratios (WT = 8.89 ± 0.58 mg/mm; HG-KO = 8.43 ± 0.62 mg/mm) from the baseline values presented in Fig. 2C; these are similar to the post-TAC values in the same figure. Doppler mitral valve inflow data from echo revealed that the HG-KO mice also exhibited a significantly ($P < 0.05$) increased peak of E wave amplitude with a shortened deceleration time as previously reported in a similar model.¹³ There were no morphometric or functional changes in sham control mice. Histological sections showed normal tissue structure for WT and HG-KO control mice (Figs. 1A & 1C) prior to ISO treatment. After ISO treatment, both WT and HG-KO mice exhibited hypertrophy, no necrosis and mild (grade 1) interstitial subendocardial fibrosis in the free wall (Figs. 1B & 1D).

Unlike the HG-KO mice, the V-KO mice were intolerant to the 0.8mg per day dose of ISO. Only one of the V-KO mice survived up to the seventh day. The two others died between the first and the fourth day. All the wild-type mice survived (4 WT, 3 V-KO mice, age 11.9 weeks). Sham controls ($n = 4$ V-KO & 2 WT; age 11.7 weeks) that received saline for 7 days all survived and exhibited no morphometric or functional differences from pre-surgical values. Due to the high mortality, we reduced the ISO dosage to 0.4mg and 0.2mg per day in an effort to determine a threshold tolerance level. The outcome was very similar in that all the V-KO mice died within 4 days ($n = 5$ WT & 4 V-KO; mean age 11.2 weeks). The histological sections from the control V-KO mice (Fig 1E, inset) exhibited mild (grade 1) fibrosis. In contrast, the heart of the V-KO mouse that survived for 7 days showed hypertrophy, with abnormal ventricles and walls,

diffuse marked (grade 3) myocardial fibrosis, a loss of myocardium with replacement calcification and (grade 3) coagulation necrosis (Fig. 1F & inset).

Telemetry Analysis

The electrocardiogram, body temperature and cage activity were monitored continuously in WT and KO mice from both genotypes for 2 to 8 weeks (Table 2). There were no differences in heart rates, body temperatures and cage activities between WT and KO mice within a genotype and diurnal cycle period. All three of these parameters were significantly higher during the active period as expected. The HG-KO mice had a slightly higher heart rate than their WT littermates, but there were no differences in waveform shape or interval durations (except R-R). Most of the V-KO mice died during the recordings between 14 to 22 weeks of age. An increasing number of arrhythmias (PVCs, conduction abnormalities) were observed in the V-KO mice a few days prior to death; no arrhythmias were observed in the HG-KO or the WT mice. The 38% shorter QT interval in the V-KO mice has been previously reported.¹⁶ Interestingly the R/P wave amplitude ratio in the V-KO mice is only 16% as large as the values of wild type littermate's during both the light and dark cycles ($P < 0.001$). Although the absolute R/P wave amplitude ratios were different between both groups, there was no difference between the HG-KO and their own wild type mice littermates.

Discussion

Alteration of Ca^{2+} homeostasis is a common finding associated with the cardiac dysfunction, hypertrophy and heart failure. The amount of NCX1 in the myocardium is important for the maintenance of Ca^{2+} homeostasis. Increased NCX1 expression is associated with maladaptive remodeling early in the transition to heart failure in both human and mouse models. It has also been reported that the inhibition of NCX1 may be cardioprotective.^{22,23} To determine the role of reduced NCX1 expression in myocardial contraction and the pathophysiology of myocardial hypertrophy, Takimoto et al.¹³ studied heterozygous global NCX1-KO mice after a three week TAC intervention. These mice are similar to our HG-KO mouse model. They concluded that the reduction of the exchanger results in compensatory increase of myocardial function even in the presence of hypertrophy after TAC. They also reported no differences in SERCA2, DHPR and RyR2 expression in the left ventricle between sham KO and WT mice. Interestingly, they reported that SERCA2 expression levels were reduced post TAC in the WT mice but did change in KO mice. Moreover DHPR expression levels were reduced in both KO and WT mice after TAC, but RyR2 levels did not change.¹³

To further investigate the role of reduced NCX1 in myocardial hypertrophy and heart failure we conducted the present study with two distinct KO genotypes using several assessment tools. The first study was conducted in our HG-KO mouse model which has about 50% of the normal expression level of NCX1 in every myocyte. These mice appear to be nearly normal in terms of baseline values and their response to TAC and β -adrenergic stimulation (Figs 1-3). Interestingly, HG-KO mice have smaller hearts with higher heart rates at 3 months of age with respect to their wild-type littermates at baseline (Tables 1 & 2, and Fig. 2). But their myocardial size normalizes to that of the wild-type by 1 year of age and their ventricular function was well preserved up to 18 months. Similar to Takimoto's mice,¹³ post-TAC remodeling was a well compensated hypertrophy without heart failure (Fig 2). Also, we observed some evidence of diastolic dysfunction in the LV pressure and Doppler echo data. Unlike Takimoto's findings, our HG-KO mice had no post-TAC increase in contractility and relaxation as compared to baseline and WT TAC. However, our baseline contractility and relaxation values for both the HG-KO and WT mice were nearly twice as great as Takimoto's (Fig 3) and within what we consider the normal physiological range. Thus, the HG-KO myocyte with only $\frac{1}{2}$ the normal NCX1 expression appears to still have the capacity to handle increased Ca^{2+} fluxes under

normal and stressed conditions. Despite being smaller in size, the HG-KO hearts are able maintain normal EF, contractility, pressures and presumably normal cardiac outputs via increased heart rates with only modest indications of diastolic dysfunction post-TAC.

Previous studies from our group have investigated the basic biochemical and electrophysiological characteristics of the V-KO mice.^{12,14-16} There is no exchanger in 80-90% of the myocytes; the remaining 10 - 20% of myocytes are normal. This is a fundamentally different paradigm of reduced expression as compared to the HG-KO model. We previously reported baseline electrophysiological abnormalities and a 21% decline in the ejection fraction in the seven week old mice. The electrophysiological experiments revealed normal Ca^{2+} transients in isolated V-KO myocytes and a normal response to isoproterenol (ISO).^{12,14-16} Voltage clamp studies revealed that the L-type Ca^{2+} channel current was reduced up to 50% even though the number of channels was not decreased. Also, the action potential was shorter which further reduces Ca^{2+} influx. ECG telemetry in V-KO mice revealed shorter QT intervals compared to their wildtype (WT) littermates (Table 2). This finding is consistent with the shorter AP duration and early repolarization found in myocytes.^{14,16,24} The V-KO mice, when young, appear to functionally adapt to the absence of the exchanger by reducing Ca^{2+} influx instead of upregulating other Ca^{2+} efflux mechanisms.

We now report that baseline echocardiographic evaluation of older V-KO mice revealed significant myocardial remodeling (Table 1 and Figs 1 & 2) with many mice dying of heart failure at around 14 weeks of age. Though one mouse lived to 22 weeks of age, ejection fractions measured from 14 week old baseline and sham V-KO mice of age were less than 50% suggesting very poor ventricular function. Another interesting feature of the V-KO mice is their blunted response of $\pm \text{dP/dT}$ to β -adrenergic stimulation in comparison to their WT littermates and the HG-KO model (Figs. 3 & 4). This should be expected, since these mice manifest hypertrophy and a reduction in LV contractility at baseline. Their myocardium may have a chronic increased stimulation of the sympathetic system in response to a downregulation of β -adrenergic receptor sensitivity or number, or by the uncoupling from G_s and an increase of G_{ai} as has been reported in heart failure.²⁵⁻²⁷ Moreover, dobutamine improves contractility by increasing intracellular concentration of free calcium, which is normally extruded by NCX1. The V-KO mice had a blunted or even depressed response below baseline to dobutamine (Fig. 4B) possibly because of a form of myocardial stunning caused by a rise of intracellular calcium which cannot be extruded normally from the majority of the myocytes due to their lack of NCX1.¹⁶ Despite the blunted contractility, the V-KO heart rates increased similar to WT mice with dobutamine even when contractility was severely blunted (Fig. 4B). Thus the SA node function is normal as expected since NCX1 is still present in the atria of all myocytes.

Chronic infusion of ISO, a non specific β -adrenergic agonist, induces early hypertrophy and maladaptive remodeling in many models of genetically manipulated mice.^{25,28,29} Prolonged activation of the sympathetic nervous system by agents such as ISO is deleterious for the myocardial function and can accelerate the transition to heart failure. Chronic stimulation with ISO in the HG-KO model was unremarkable leading only to the development of mild fibrosis similar to their WT littermates (Figs. 1A-D). On the contrary, chronic stimulation with ISO in V-KO mice was associated with high mortality due to heart failure and profound histological changes (Figs. 1E & 1F).

Thus, stressing the V-KO mice with TAC or β -adrenergic agents confirmed that a severe reduction in the number of myocytes expressing the exchanger gives very little myocardial plasticity to adapt to situations in which more Ca^{2+} efflux is required. Similar to the mice overexpressing NCX1,⁸ a profound reduction in the number of myocytes with NCX1 contributes to the transition to heart failure because in both cases the calcium homeostasis is perturbed in the myocardium. In the V-KO mice, there is insufficient exchanger capacity to

remove the excess calcium as the demands upon the heart increase with age or with additional stress from TAC and β -adrenergic stimulation. Sham controls were well tolerated in both models with no change in morphometry and function from baseline.

The telemetric analyses (Table 2) revealed increased heart rates, cage activity and body temperatures during the dark (active) cycle as expected from the mice responding to their normal diurnal rhythms. Of more interest are the changes in wave amplitudes and intervals which provide some insight into the effect of altered calcium homeostasis upon the ECG. We previously reported that the V-KO mice have shorter QT intervals corresponding to their shorter action potentials.¹⁶ This 38% shorter interval along with the increased fibrosis and necrosis likely contribute to the arrhythmias (ventricular fibrillation, bigemini, conduction abnormalities or PVC complexes) seen in older V-KO mice at baseline and following low dose dobutamine administration (Figures 4A & 4B). The reduction of NCX1 and/or shortened QT intervals have been shown to be arrhythmogenic in human and other animal models.^{30,31} Since the P and R wave amplitudes reflect the respective summation of action potential changes in the atria and ventricular myocytes, the R/P ratio is of interest. We found that the R/P wave amplitude ratio in V-KO mice is about 1/6 as much as those measured in their WT littermates (Table 1). All atrial cells in this model should have normal action potentials that contribute to the P wave amplitude of the ECG. However, only 10-20 % of the V-KO ventricular myocytes have normal action potentials that contribute to the R wave amplitude. The KO myocytes with no exchanger have narrow action potentials that apparently contribute less to the R wave amplitude. This, along with the structural alterations from increased fibrosis and necrosis, are the most likely contributing factors to the R/P ratio difference.

In summary the 50% reduction of the exchanger in all the myocytes of HG-KO mice is associated with normal myocardial structure and function at baseline, tolerance to pressure overload with hypertrophy, but no transition to heart failure and a preserved responsiveness to β -adrenergic stimulation. The 80 % reduction of myocytes expressing NCX1 in V-KO mice is associated with decreased myocardial function, hypertrophy leading to heart failure, marked myocardial injury, alterations in the ECG, and poor tolerance to hemodynamic and pharmacological stress. The severe reduction of myocytes expressing the exchanger in the V-KO mice does not give the myocardium the ability to compensate to increased levels of calcium fluxes associated with growth to adulthood and under hemodynamic stress. The milder global reduction of NCX1 in all the HG-KO myocytes appear to be sufficient to give their myocardium adequate plasticity to adapt to different conditions of Ca^{2+} efflux as opposed to the more compromised V-KO mouse model. Thus there appear to be no significant beneficial, only neutral or detrimental effects of reduced NCX1 to cardiac function.

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Fig. 1. Myocardial Morphology

Sections from the posterior (lateral) free wall of the left ventricular were taken after chronic ISO infusion for 7 days in a V-KO and HG-KO mice and their WT littermates. Panels A & B are the pre- and post infusion samples from WT mice; panels C & D are the pre- and post infusion samples from HG-KO mice; and panels E & F are the pre- and post infusion samples from V-KO mice. All panels are trichrome stain; magnification for all is 40X with the insets at 200X. See text for details.

2A

2B

2C

Fig. 2. Baseline and Post-TAC Remodeling and Function

Baseline, sham and post-TAC remodeling is assessed in terms of ventricular septal thickness (VST, mm) in panel A, left ventricular mass (LVM, mg) in panel B and heart weight to tibia length ratio (HW/TL, mg/mm) in panel C. Changes in ventricular function in terms of ejection fraction (EF) are shown in panel D. * indicates significance ($P < 0.05$) between WT and KO mice within the same model. † indicates significance ($P < 0.05$) between the pre- and post-sham or post-TAC mice within the same model. N values for each group are listed above the plots. The X in figure panels A, B, and D indicate that no V-KO mice survived the full 21 days for post-TAC echo evaluation.

3B

3C

3D

Fig. 3. Response to β -adrenergic Stimulation

Indices of (A) contractility (+dP/dt) and (B) relaxation (-dP/dt) are plotted versus dose (1-30 ng/Kg) of bolus infusions of dobutamine for both groups of mice. Similarly, the indices of (C) contractility (+dP/dt) and (D) relaxation (-dP/dT) are plotted versus dose (0.05-1 ng/Kg) of bolus infusions of isoproterenol for the V-KO mice. Baseline indicates the stable value taken prior to the series of infusions. * indicates significance ($P < 0.05$) between WT and KO mice within the same model.

Fig. 4. Arrhythmias and Dysfunction in V-KO Mice

Panel A shows an episode of ventricular fibrillation and recovery (bar) prior to dobutamine infusion in one mouse. Panel B illustrates an entire dobutamine infusion study during which there was a depressed pressure and contractility response at the time of maximal heart rates at the higher doses (arrows). Panel C illustrates a similar dobutamine study with a WT mouse with normal responses.

Table 1
LV Chamber Dimensions and Function from Echo

	HG-KO		V-KO	
	WT	KO	WT	KO
Age in weeks	11.5 ± .6	11.5 ± .9	10.5 ± .5	10.7 ± .4
N value	8	9	6	12
Body Weight (g)	28.2 ±1.5	25.8 ±1.3	23.8 ±1.3	24.5 ±0.9
Ventricular Septal Thickness (mm)	0.54 ± .02	0.47* ± .02	0.52 ± .02	0.61* [†] ± .02
Posterior Wall Thickness (mm)	0.51 ± .03	0.46 ± .02	0.50 ± .02	0.61* [†] ± .03
End Diastolic Dimension (mm)	4.00 ± .09	3.94 ± .08	3.87 ± .07	4.02 ± .12
End Systolic Dimension (mm)	2.36 ± .11	2.53 ± .06	2.25 ± .11	3.02** [†] ± .14
LV Mass (mg)	69 ± 3	57* ± 2	62 ± 3	84* [†] ± 6
LV Fractional Shortening (%)	41.1 ± 2.0	35.6 ± 1.6	41.9 ± 2.0	25.2** [†] ± 2.2
VCF (mm/sec)	7.48 ± .45	6.32 ± .23	7.36 ± .57	5.04* ± .40
LV Ejection Fraction (%)	76.9 ± 2.3	71.6 ± 1.8	79.4 ± 1.8	54.8** [†] ± 3.7

Significance:

* = P < 0.05 to WT value of the same group

** = P < 0.001 to WT value of the same group

[†] = P < 0.05 to HG-KO

Table 2

Telemetric Analysis

	HG-KO		V-KO	
	WT	KO	WT	KO
Age (weeks) (at end)	22.3 ± .8	18.5 ± .6	17.6 ± .9	16.0 ±1.4
N (mice)	5	5	8	8
<u>Light Cycle Values</u>				
Heart Rate (bpm)	576 ±13	584 ±3	529 ±3	528 [†] ±18
Cage Activity (Arbitrary Units)	5.6 ±0.7	3.3 ±0.4	4.0 ±0.4	5.2 ±1.1
Body Temp (C°)	36.1 ±.1	36.3 ±.1	36.5 ±.1	36.6 ±.1
PR Interval (ms)	37.9 ±0.9	36.7 ±1.1	39.2 ±0.7	37.8 ±1.1
QT Interval (ms)	45.8 ±2.2	48.6 ±3.9	51.7 ±1.8	38.0 ^{**†} ±2.2
R/P Ratio	11.4 ±1.9	9.8 ±1.2	17.4 ±2.0	3.0 ^{**†} ±0.8
<u>Dark Cycle Values</u>				
Heart Rate (bpm)	639 [‡] ±14	646 [‡] ±10	554 ^{†‡} ±8	590 ^{†‡} ±12
Cage Activity (Arbitrary Units)	17.7 [‡] ±2.5	11.6 [‡] ±2.4	9.3 [‡] ±1.7	11.2 [‡] ±1.9
Body Temp (C°)	37.2 [‡] ±.1	37.3 [‡] ±.2	37.3 [‡] ±.1	37.6 [‡] ±.2
PR Interval (ms)	37.1 ±0.8	36.8 ±1.4	38.4 ±0.7	36.7 ±0.8
QT Interval (ms)	44.8 ±2.6	49.2 ±3.8	51.2 ±2.1	36.7 ^{**†} ±2.1
R/P Ratio	11.2 ±1.5	10.9 ±1.9	17.9 ±2.0	2.7 ^{**†} ±0.6

Significance:

** = P < 0.001 to WT value of the same group

† = P < 0.05 to HG-KO

‡ = P < 0.05 to corresponding light cycle value