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No Contribution of IP₃-R(2) to Disease Phenotype in Models of Dilated Cardiomyopathy or Pressure Overload Hypertrophy

Nicola Cooley, PhD*; Kunfu Ouyang, PhD*; Julie R. McMullen, PhD; Helen Kiriazis, PhD; Farah Sheikh, PhD; Wei Wu, PhD; Yongxin Mu, PhD; Xiao-Jun Du, MD, PhD; Ju Chen, PhD; Elizabeth A. Woodcock, PhD

Background—We investigated the contribution of inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors (IP₃-R) to disease progression in mouse models of dilated cardiomyopathy (DCM) and pressure overload hypertrophy. Mice expressing mammalian sterile 20-like kinase and dominant-negative phosphatidylinositol-3-kinase in heart (Mst1^{xdn}-PI3K-2Tg; DCM-2Tg) develop severe DCM and conduction block, associated with increased expression of type 2 IP₃-R (IP₃-R(2)) and heightened generation of Ins(1,4,5)P₃. Similar increases in Ins(1,4,5)P₃ and IP₃-R(2) are caused by transverse aortic constriction.

Methods and Results—To evaluate the contribution of IP₃-R(2) to disease progression, the DCM-2Tg mice were further crossed with mice in which the type 2 IP₃-R (IP₃-R(2)^{-/-}) had been deleted (DCM-2Tg×IP₃-R(2)^{-/-}) and transverse aortic constriction was performed on IP₃-R(2)^{-/-} mice. Hearts from DCM-2Tg mice and DCM-2Tg×IP₃-R(2)^{-/-} were similar in terms of chamber dilatation, atrial enlargement, and ventricular wall thinning. Electrophysiological changes were also similar in the DCM-2Tg mice, with and without IP₃-R(2). Deletion of IP₃-R(2) did not alter the progression of heart failure, because DCM-2Tg mice with and without IP₃-R(2) had similarly reduced contractility, increased lung congestion, and atrial thrombus, and both strains died between 10 and 12 weeks of age. Loss of IP₃-R(2) did not alter the progression of hypertrophy after transverse aortic constriction.

Conclusions—We conclude that IP₃-R(2) do not contribute to the progression of DCM or pressure overload hypertrophy, despite increased expression and heightened generation of the ligand, Ins(1,4,5)P₃. (*Circ Heart Fail.* 2013;6:318-325.)

Key Words: atrium ■ dilated cardiomyopathy ■ echocardiography ■ experimental models of heart failure ■ pressure overload

The failing myocardium from humans and experimental animals commonly shows increased expression of the receptors for the Ca²⁺-regulating second messenger inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors (IP₃-R), often along with a decrease in ryanodine receptors, the master regulators of Ca²⁺ within the myocardium.¹ The importance, or otherwise, of this has been debated. The altered relative expression levels of the intracellular Ca²⁺ channels might reflect a degree of dedifferentiation of the failing ventricular myocytes, with no major functional implications. Alternatively, increased IP₃-R might contribute to, or ameliorate, the progression of heart failure. IP₃-R have been suggested to contribute to cardiomyocyte hypertrophy,^{2,3} chamber dilatation,⁴ and arrhythmogenesis,⁵ but there is little agreement between laboratories in relation to any of these claims.

Under physiological conditions, cardiomyocytes from all species studied show little or no change in Ins(1,4,5)P₃ in response to the activation of appropriately coupled receptors,⁶ and, in addition, the expression of IP₃-R is low.⁷ These findings argue against a strong involvement of IP₃-R under physiological conditions. However, our earlier and recent studies show that generation of Ins(1,4,5)P₃ is markedly heightened under pathological conditions such as atrial dilatation in humans, mice with dilated cardiomyopathy (DCM),⁴ and in hypertrophied mouse ventricle after chronic pressure overload.⁸ Furthermore, we have observed heightened expression of phospholipase Cβ1b, the enzyme responsible for generation of Ins(1,4,5)P₃ in myocytes, from failing human, mouse, and sheep myocardium⁴ (sheep data not shown). All these findings suggest that Ins(1,4,5)P₃ may be of heightened importance in failing myocardium, especially given the likelihood of increased IP₃-R expression.^{7,9}

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We undertook an investigation of the possible pathological roles of IP₃-R using a mouse model with severe DCM and clear evidence of conduction block (DCM-2Tg), as well as a well-characterized model of pressure overload hypertrophy, induced by transverse aortic constriction (TAC).⁸ Both of these murine models show increased generation of Ins(1,4,5)P₃^{4,8} and increased expression of type 2 IP₃-R (IP₃-R(2)), the IP₃-R subtype expressed in working cardiomyocytes. To examine possible contributions of the heightened Ins(1,4,5)P₃ and IP₃-R(2) to hypertrophy and dilatation, we performed TAC on IP₃-R(2)^{-/-} mice and crossed the DCM-2Tg with IP₃-R(2)^{-/-} mice and examined the effect on progression of disease in both scenarios.

Methods

Experimental Animals

All experiments were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee.

DCM-2Tg Model

Cardiac-specific Mst1 (line no. 28; C57BL/6 background) and dnPI3K (FVB/N) transgenic mice were generated and genotyped as described previously.^{10,11} IP₃-R(2)^{-/-} mice were generated on a C57BL/6 background, as described.⁵ Male heterozygous Mst1-Tg (Mst1^{+/-}) were genetically crossed with dnPI3K^{+/-} and IP₃-R(2)^{-/-} to generate DCM-2Tg mice (Mst1^{+/-}dnPI3K^{+/-}, Mst1×dnPI3K-2Tg, DCM-2Tg) and DCM-2Tg-knockout mice (Mst1^{+/-}dnPI3K^{+/-}IP₃-R(2)^{-/-}).¹² The crossed strain (Mst1×dnPI3K-2Tg) was chosen for these studies because, unlike the Mst1-Tg, they show severe conduction block and we were interested in possible atrial actions of IP₃-R(2). All mice were bred on the same mixed genetic background (C57BL/6/FVB/N).

Transverse Aortic Constriction

Two-month-old male mice were anesthetized with ketamine/xylazine and underwent a sham operation or were subjected to pressure overload induced by TAC as previously described.¹³ Echocardiography measurements were performed before surgery and at 1 and 4 weeks after TAC. The pressure gradients generated by aortic banding were measured by introducing high-fidelity pressure transducers into the left and right common carotids. Pressure gradients in IP₃-R(2)^{-/-} and wild-type (WT) littermates were similar (Figure 1A in the online-only Data Supplement). Hearts were collected and morphometrics evaluated 4 weeks post-TAC.

Transthoracic Echocardiography and Electrocardiography

Anesthesia was maintained with 1.7% isoflurane. Echocardiography was performed using a Philips iE33 ultrasound machine and a 15-MHz linear transducer. Electrocardiography (ECG) recordings were measured using the Powerlab System and BioAmp (ADInstruments). Data were measured digitally using ADInstruments (Chart 5 Pro ECG analysis module). Details are provided in the online-only Data Supplement.

Measurement of Ins(1,4,5)P₃ and Its Metabolites in Atria and Ventricles (Phospholipase C Activation)

Tissues were labeled with [³H]inositol and subsequently stimulated with norepinephrine (50 μmol/L). [³H]Ins(1,4,5)P₃ and its metabolites were extracted and quantified by high-performance liquid chromatography, as described previously.^{4,14} Details are provided in the online-only Data Supplement.

Measurement of mRNA and Protein Expression in Atria and Ventricles

RNA was extracted using RNEasy kits from Qiagen or Trizol reagent (Invitrogen) according to the manufacturer's instructions and reverse transcribed using Superscript III (Invitrogen). Quantitative real-time polymerase chain reaction with SybrGreen (Invitrogen) reagent was performed on an Applied Biosciences 7500 Fast Real-Time PCR System. Primer sequences are provided in the online-only Data Supplement. Values are expressed as the 2^{-ΔCt} value relative to GAPDH in each sample.¹⁵ All quantitative real-time polymerase chain reaction experiments were performed in triplicate on triplicate or quadruplicate samples. RNA dot-blot analyses were performed as previously described.¹⁶ Protein methods are outlined in the online-only Data Supplement.

Data Evaluation

For experiments involving 2 groups (WT and DCM or sham and TAC), or where n=3, data were analyzed using a Mann-Whitney rank-sum test. For experiments involving 4 groups (WT, IP₃-R(2)^{-/-}, DCM, DCM×IP₃-R(2)^{-/-} or Sham, sh. IP₃-R(2)^{-/-}, TAC, TAC IP₃-R(2)^{-/-}), a 2-way ANOVA was used to assess the effect of TAC or DCM, the IP₃-R(2) status, and the interaction between the 2 (Sigma Stat). Where repeated measures were involved (echo measurements after TAC), a repeated measures 2-way ANOVA was used. All pairwise multiple comparisons used a Holm-Sidak post hoc test, except where normality tests failed and a rank-sum test was used (Sigma Stat).

Results

Hearts From DCM-2Tg Mice or TAC Mice Have Heightened Expression of IP₃-R(2) and Heightened Generation of Ins(1,4,5)P₃

The IP₃-R(2) is expressed in working cardiomyocytes, and we measured expression in hearts from DCM-2Tg mice that have DCM, as well as from mice that have undergone TAC to induce pressure overload hypertrophy. The DCM-2Tg mice had heightened IP₃-R(2) expression in atria and left ventricles (Figure 1A). Loss of IP₃-R(2) in IP₃-R(2)^{-/-} mice did not result in altered expression of either of the other IP₃-R subtypes, IP₃-R(1) or IP₃-R(3) (Figure I in the online-only Data Supplement). IP₃-R(2) expression also was substantially elevated 4 weeks after TAC (Figure 1B and 1C).

We have previously reported that TAC-induced hypertrophy leads to heightened InsP responses in mouse hearts, resulting in increased generation of Ins(1,4,5)P₃.⁸ In the current study, we assessed Ins(1,4,5)P₃ generation in atria and ventricles from DCM-2Tg and littermate WT mice. This was accomplished by labeling the inositol phospholipids with [³H]inositol and subsequently measuring the generation of [³H]-labeled Ins(1,4,5)P₃ and its metabolites as a measure of total [³H]Ins(1,4,5)P₃ generation. As described previously,⁴ atria and ventricles from WT mice have low levels of [³H]InsPs after 20 minutes of treatment with norepinephrine (50 μmol/L), indicating minimal generation of Ins(1,4,5)P₃. Responses were substantially heightened in atria and ventricles from DCM-2Tg mice, reflecting increased Ins(1,4,5)P₃ production (Figure 1D).

Deletion of IP₃-R(2) Does Not Influence Heart Size in DCM-2Tg Mice or TAC Mice

DCM-2Tg mice develop DCM, indicated by chamber dilatation, left ventricular wall thinning, and decreased contractile function.¹² We argued that heightened generation

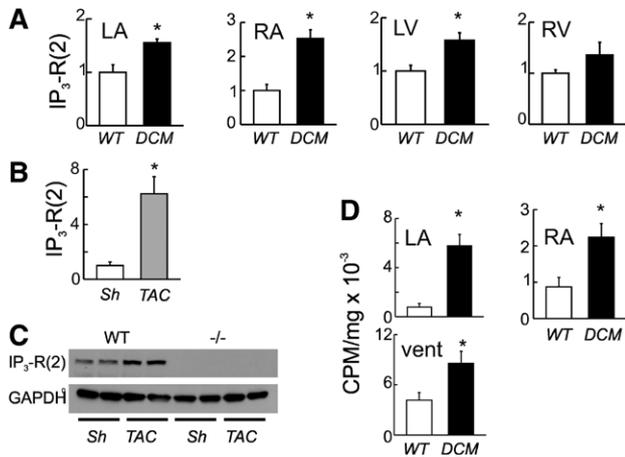


Figure 1. Hearts of dilated cardiomyopathy (DCM)-2Tg mice and transverse aortic constriction (TAC) mice have heightened type 2 inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptor (IP₃-R(2)) expression and InsP generation. **A**, Expression of IP₃-R(2) in atria and ventricles from DCM-2Tg mice and littermate controls (wild type [WT]). Values shown are IP₃-R(2) mRNA expressed as fold change, mean±SEM (n=5–7). **B**, Expression of IP₃-R(2) (mRNA relative to GAPDH) in left ventricle of mice 4 weeks after TAC. **C**, Expression of IP₃-R(2) at the protein level 4 weeks after TAC. The experiment was performed 3× with similar results. **D**, Generation of [³H]Ins(1,4,5)P₃ and its metabolites in atria and ventricles from WT and DCM-2Tg mice. Values shown are [³H]inositol phosphates, counts per min/mg of tissue, mean±SEM (n=5–7); *P<0.05 relative to WT or sham (Sh)-operated control (Mann-Whitney rank-sum test). LA indicates left atrium; LV, left ventricle; RA, right atrium; and RV, right ventricle.

of Ins(1,4,5)P₃, together with heightened expression of IP₃-R(2), might contribute substantially to the DCM phenotype in these animals. To test this possibility, we examined the phenotype of the DCM-2Tg mice in which IP₃-R(2) had been deleted (DCM-2Tg×IP₃-R(2)-/-) and compared this with DCM mice expressing the endogenous complement of IP₃-R(2) (DCM-2Tg). As reported previously,¹² DCM mice exhibited severe left ventricular dilatation (Figure IIB in the online-only Data Supplement and Table 1), increased atrial size, and ventricular wall thinning compared with WT mice

(Table 1). There was no hypertrophy observed in the DCM-2Tg hearts. DCM-2Tg mice with IP₃-R(2) deleted showed a phenotype indistinguishable from that of DCM-2Tg mice (Tables 1 and 2).

In contrast to the DCM-2Tg model, mice subjected to TAC develop ventricular hypertrophy indicated by increased left ventricle/body weight ratio and increased ventricular wall thickness. Deletion of IP₃-R(2) did not alter the cardiac phenotype either in TAC mice or in sham-operated controls. Measurements were made both early (1 week) and late (4 weeks after TAC), and no effect of IP₃-R(2) deletion was observed at either time point (Figure 2).

Deletion of IP₃-R(2) Does Not Alter Gene Expression Profiles in DCM-2Tg Mice or TAC Mice

DCM-2Tg mice show alterations in ventricular gene expression, reflecting disease progression.¹² We examined transcriptional responses in DCM mice with and without their complement of IP₃-R(2). As shown in Figure 3A, DCM in the DCM-2Tg was associated with increased expression of atrial natriuretic peptide and α-skeletal actin. Deletion of IP₃-R(2) did not alter these expression changes. As reported previously,⁸ TAC induced changes in hypertrophic gene expression profiles, atrial natriuretic peptide, α-myosin heavy chain, β-myosin heavy chain, and α-skeletal actin, and these were not altered by deletion of IP₃-R(2) (Figure 3C and 3D).

Ins(1,4,5)P₃/IP₃-R(2) signaling has been suggested to contribute to hypertrophy by generating the Ca²⁺ required to activate calcineurin, which in turn dephosphorylates the nuclear factor of activated T cells family of transcription factors culminating in altered gene expression profiles.¹⁷ Calcineurin is known to be activated by TAC, and we examined whether deletion of IP₃-R(2) altered calcineurin signaling after TAC. Modulatory calcineurin-interacting protein is a direct transcriptional target of calcineurin, and we measured modulatory calcineurin-interacting protein as an index of calcineurin signaling. As shown in Figure 3B, modulatory calcineurin-interacting protein expression was increased at 4

Table 1. Echocardiographic Parameters in DCM-2Tg mice and the Effect of Deletion of IP₃-R(2)

	WT (7)	IP ₃ -R(2)-/- (5)	DCM-2Tg (6)	DCM-2Tg×IP ₃ -R(2)-/- (12)	P		
					DCM	IP ₃ -R(2)-/-	Int.
Sex, male/female	5/2	4/1	2/4	5/7			
Age, wk	9.1±1.4	7.2±0.5	8.0±0.1	8.8±0.7			
HR, bpm (echo)	571±30	628±48	490±34	507±20	NS	ns	ns
LVPWs, mm	1.3±0.10	1.2±0.05	0.75±0.05	0.8±0.05	<0.001	0.619	0.943
LVPWd, mm	0.92±0.09	0.94±0.06	0.66±0.04	0.68±0.05	<0.001	0.516	0.201
LVIDs, mm	1.97±0.18	2.2±0.26	3.69±0.14	3.7±0.18	<0.001	0.324	0.203
LVIDd, mm	3.5±0.17	3.4±0.2	4.2±0.18	4.3±0.16	<0.001	0.567	0.660
IVSs, mm	1.53±0.11	1.4±0.12	0.79±0.06	0.87±0.06	<0.001	0.762	0.181
IVSd, mm	0.96±0.07	1.02±0.04	0.68±0.04	0.71±0.04	<0.001	0.373	0.830
LA area, mm ²	7.6±0.58	7.24±0.9	21.7±2.3	19.68±1.6	<0.001	0.593	0.637

Values are mean±SEM. The number of animals is indicated in brackets. Data were analyzed by 2-way ANOVA, and P values shown are for the effect of DCM, effect of IP₃-R(2)-/-, and the interaction (int.) between DCM and IP₃-R(2)-/-. bpm indicates beats per minute; DCM, dilated cardiomyopathy; HR, heart rate; IP₃-R(2), type 2 inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors; IVSd, intraventricular septum diastole; IVSs, intraventricular septum systole; LA, left atrium; LVIDd, LV internal diameter diastole; LVIDs, LV internal diameter systole; LVPWd, LV posterior wall diastole; LVPWs, LV posterior wall systole; NS, not significant; and WT, wild type.

Table 2. Morphometric Parameters in DCM-2Tg Mice and the Effect of Deletion of IP₃-R(2)

	WT (6)	IP ₃ -R(2) ^{-/-} (6)	DCM-2Tg (12)	DCM-2Tg×IP ₃ -R(2) ^{-/-} (18)	<i>P</i>		
					DCM	IP ₃ -R(2) ^{-/-}	Int.
Sex, men/women	4/2	4/2	5/7	8/10			
Age, wk	13±0.58	10±0.75	10.5±1.3	11.8±1.4			
Ventricle/tibia, mg/mm	7.05±0.17	6.85±0.41	6.41±0.41	6.47±0.30	NS	NS	NS
Atria/tibia, mg/mm	0.39±0.09	0.37±0.05	0.94±0.2	0.88±0.11	<0.001	0.486	0.294
Body weight, g	28.2±2.4	31.4±3.8	23.8±1.6	26.8±1.3	NS	NS	NS

Values are mean±SEM. The number of animals is indicated in brackets. Data were analyzed by 2-way ANOVA, and *P* values shown are for the effect of DCM, effect of IP₃-R(2)^{-/-}, and the interaction (int.) between DCM and IP₃-R(2)^{-/-}. DCM indicates dilated cardiomyopathy; IP₃-R(2), type 2 inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors; NS, not significant; and WT, wild type.

weeks after TAC, but deletion of IP₃-R(2) did not influence expression either in TAC or in sham-operated mice.

Deletion of IP₃-R(2) Does Not Alter ECG Profiles in DCM-2Tg Mice

We next examined whether deletion of IP₃-R(2) influenced electrophysiological changes that we have previously found to be associated with heart failure in the DCM model.¹² No arrhythmias were observed over the time periods studied. Relative to WT littermates, ECG profiles from DCM-2Tg mice showed prolonged P-R interval, prolonged Q-T interval, and reduced R amplitude. R-R interval, QRS interval, and ST height were not significantly altered in the DCM mice. Deletion of IP₃-R(2) did not influence these changes, and data obtained from DCM-2Tg and DCM-2Tg×IP₃-R(2)^{-/-} were not different in terms of any of these parameters. Deletion of IP₃-R(2) also did not alter ECG profiles in the absence of DCM (Figure IIC in the online-only Data Supplement and Table 3).

Deletion of IP₃-R(2) Does Not Alter Functional Parameters in DCM-2Tg Mice or in TAC Mice

DCM-2Tg mice die prematurely by 10 to 12 weeks of age. Deletion of IP₃-R(2) did not alter the death rate in this strain (Figure 4A). DCM-2Tg mice showed a high incidence of pleural effusion, lung congestion, and atrial thrombus, consistent

with severe heart failure. Deletion of IP₃-R(2) did not alter any of these parameters (Figure 4B). Contractility, measured as fractional shortening by echocardiography, was substantially reduced in the DCM-2Tg mice, but was not influenced by deletion of IP₃-R(2) (Figure 4C and Table 1). Lung congestion was similar in DCM-2Tg mice with and without their complement of IP₃-R(2) (Figure 4D). Thus, IP₃-R(2) do not contribute substantially to the heart failure phenotype in this model.

TAC resulted in a progressive decrease in fractional shortening that was not altered by IP₃-R(2) deletion (Figure 4E). Similarly, increases in left ventricular weight were similar in WT and IP₃-R(2)^{-/-} mice after TAC (Figure 4F).

Discussion

Heart failure in humans and experimental animals has repeatedly been reported to be associated with increased ventricular expression of IP₃-R(2) in the left ventricle.^{7,18,19} Increased atrial expression has been reported in valvular heart disease and atrial fibrillation.⁹ Furthermore, increased IP₃-R(2) expression has been reported in neonatal rat cardiomyocytes undergoing hypertrophy.² In some cases, the increased IP₃-R(2) expression is paralleled by a lowering of ryanodine receptor expression.^{7,18} These findings might suggest that IP₃-R(2) are of increased importance in hypertrophic and failing myocardium. It has been suggested that the increased IP₃-R activity

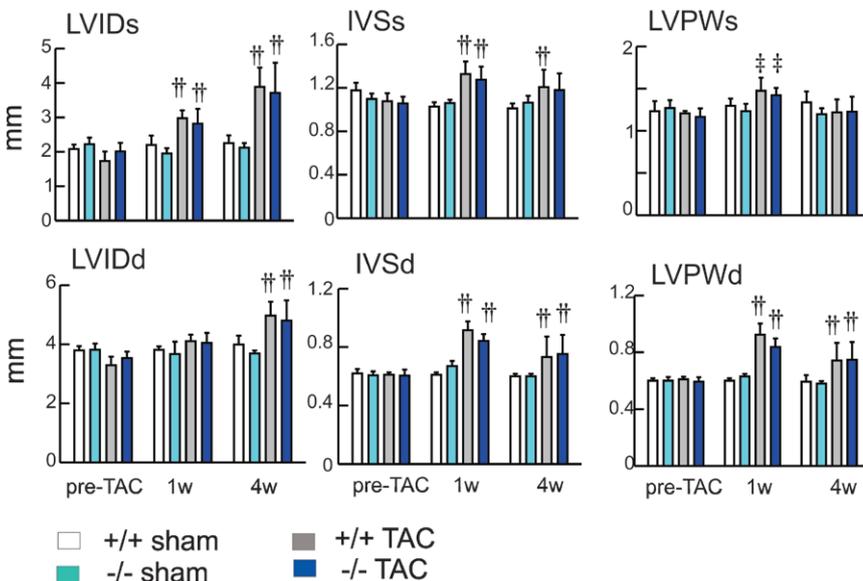


Figure 2. Deletion of type 2 inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors (IP₃-R(2)) does not alter hypertrophic responses at 1 or 4 weeks after transverse aortic constriction (TAC). Echocardiographic parameters measured in IP₃-R(2)^{-/-} mice and littermate controls (WT, +/+) before TAC and at 1 and 4 weeks after TAC. Values shown are mean±SEM (n=6 for sham groups, 13 for +/+ TAC, and 15 for -/- TAC). Analyses used a 2-way repeated measures ANOVA, as described in the Methods section. Significant effect of TAC, relative to sham; ††*P*<0.001, ‡*P*<0.01, and †*P*<0.05. There was no significant effect of IP₃-R(2)^{-/-} on any parameter, irrespective of the TAC status. IVSd indicates intraventricular septum diastole; IVSs, intraventricular septum systole; LVIDd, LV internal diameter diastole; LVIDs, LV internal diameter systole; LVPWd, LV posterior wall diastole; and LVPWs, LV posterior wall systole.

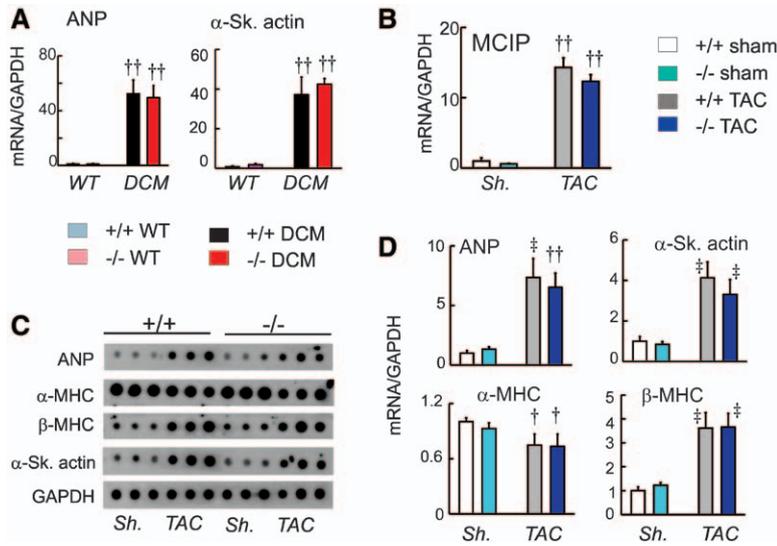


Figure 3. Lack of effect of inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors (IP₃-R(2)-/-) on transcriptional changes in dilated cardiomyopathy (DCM-2Tg) mice or transverse aortic constriction (TAC) mice. **A**, Expression of hypertrophic marker genes in left ventricle from the DCM-2Tg and wild-type (WT) mice with and without IP₃-R(2). Values shown are mRNA/GAPDH, fold change, mean±SEM (n=4–6). **B**, Expression of modulatory calcineurin-interacting protein (MCIP) in left ventricle from sham-operated and TAC mice 4 weeks after surgery (n=6). **C**, Dot blots showing mRNA expression in left ventricle from sham-operated and TAC mice with (+/+) or without IP₃-R(2) (-/-). **D**, Quantification of the data shown in (C). Values shown are mRNA/GAPDH, fold change, mean±SEM (n=3). ††P<0.001, ‡P<0.01, and †P<0.05 relative to WT or sham. There was no significant effect of IP₃-R(2)-/- on any parameter, irrespective of the DCM-2Tg or TAC status, and there was no interaction detected between IP₃-R(2)-/- and DCM-2Tg or TAC. ANP indicates atrial natriuretic peptide; MHC, myosin heavy chain; and α-Sk, α-skeletal.

contributes to pathology by promoting hypertrophic growth of the cardiomyocytes^{3,20} or that the increased IP₃-R might contribute to arrhythmia by perturbing Ca²⁺ responses close to sarcolemmal Ca²⁺ channels and the Na⁺/Ca²⁺ exchanger.^{5,21} Alternatively, the changes might reflect a loss of muscle phenotype because the cardiomyocytes undergo the transition to failure and have little functional consequence. We recently reported that dilated atrial tissues from patients with valvular heart disease have substantially heightened generation of Ins(1,4,5)P₃ and, furthermore, that generation correlated with atrial volume, suggesting a relationship to atrial dilatation. Similar observations were made in the dilated atria from hearts of mice overexpressing Mst1, where phospholipase C activity correlated with atrial weight.⁴

Transgenic mice expressing Mst1 in heart have been previously reported to have DCM,¹⁰ and the phenotype is exacerbated by coexpression of dominant-negative PI3-kinase.¹² The crossed strain (Mst1×dn-PI3K-2Tg; DCM-2Tg) has a worsened heart failure phenotype compared with Mst1-Tg and, in addition, shows clear evidence of conduction block, reflected by increased P-R interval¹² (Table 3). Both the Mst1-Tg and Mst1×dn-PI3K-2Tg strains express heightened levels of IP₃-R(2) in atria and ventricles, and additionally both

have substantially increased Ins(1,4,5)P₃ generation (Figure 1).⁴ We reasoned that the heightened Ins(1,4,5)P₃/IP₃-R axis in this mouse strain provided an excellent opportunity to evaluate its contribution to the DCM phenotype. The crossed strain (Mst1×dn-PI3K-2Tg; DCM-2Tg) was chosen for these studies because we were interested in a possible contribution of Ins(1,4,5)P₃ and IP₃-R(2) to conduction block, as well as to chamber dilatation and heart failure. The DCM model involves chamber dilatation, possibly related to enhanced cardiomyocyte apoptosis, without cardiomyocyte hypertrophy.¹⁰ In addition to a contribution to heart failure, IP₃-R have been suggested to contribute to hypertrophic signaling pathways by supplying Ca²⁺ to activate the calcineurin/nuclear factor of activated T cells (NFAT) pathway³ or by activating calcium calmodulin-dependent protein kinase II (CaMKII) localized close to the nuclear membrane.²⁰ To evaluate possible contributions of the Ins(1,4,5)P₃/IP₃-R(2) axis to hypertrophy, we used a well-established pressure overload model.²²

When we compared the DCM-2Tg mice with DCM-2Tg mice lacking IP₃-R(2), we found no difference in the extent of dilatation, chamber size, or contractile dysfunction. Indices of heart failure, lung congestion, atrial thrombus, and pleural effusion also were similar between the 2 experimental groups.

Table 3. ECG Parameters in DCM-2Tg Mice and the Effect of Deletion of IP₃-R(2)

	WT (8)	IP ₃ -R(2)-/- (5)	DCM-2Tg (8)	DCM-2Tg×IP ₃ -R(2)-/- (13)	P		
					DCM	IP ₃ -R(2)-/-	Int.
Sex, males/females	5/3	3/2	4/4	5/8			
Age, wk	9.3±1	10±3	9.2±0.8	11.4±1.6			
R amplitude, mV	1.26±0.2	1.15±0.16	0.66±0.09	0.86±0.11	<0.001	0.252	0.950
P amplitude, mV	0.13±0.01	0.16±0.01	0.057±0.01	0.048±0.01	<0.001	0.374	0.500
P-R interval, ms	37±1	35±2	54±4	56±3	<0.001	0.825	0.485
Q-T interval, ms	17±1.5	14±1	42±5	39±3	<0.001	0.482	0.239
QRS interval, ms	8±1	8±1	10±1	10±1	NS	NS	NS
R-R interval, ms	112±7	111±4	119±5	124±2	NS	NS	NS

Values are mean±SEM. The number of animals is indicated in brackets. Data were analyzed by 2-way ANOVA, and P values shown are for the effect of DCM, effect of IP₃-R(2)-/- and the interaction (int.) between DCM and IP₃-R(2)-/-. DCM indicates dilated cardiomyopathy; ECG, electrocardiography; IP₃-R(2), type 2 inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors; NS, not significant; and WT, wild type.

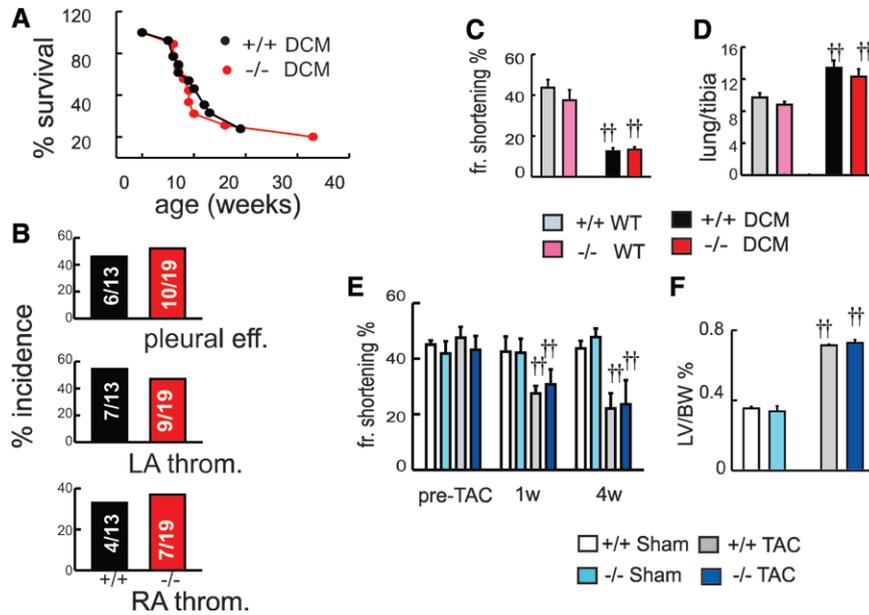


Figure 4. Deletion of type 2 inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors (IP₃-R(2)) does not alter the phenotype of the dilated cardiomyopathy (DCM)-2Tg mice or transverse aortic constriction (TAC) mice. **A**, Kaplan-Meier survival curves of DCM-2Tg mice, 10 total (black symbols), and DCM-2Tg×IP₃-R(2)^{-/-} mice, 10 total (red symbols). There was no detectable difference between death rates. **B**, Incidence of pleural effusion, left atrium (LA) and right atrium (RA) thrombus in DCM-2Tg mice with (+/+) and without (-/-) their complement of IP₃-R(2). Number of positive animals relative to the total animal number is shown on the bars. The DCM-2Tg +/+ group contained 7 of 13 females and the -/- group contained 10 of 19 females. None of the values was significantly different between the 2 groups. **C**, Fractional shortening, measured by echocardiography, in DCM-2Tg mice and DCM-2Tg×IP₃-R(2)^{-/-} mice. Values shown are mean±SEM (n=6 for the DCM-2Tg group and 12 for the DCM-2Tg×IP₃-R(2)^{-/-} group). ††P<0.001 relative to wild-type (WT) or IP₃-R(2)^{-/-} mice. **D**, Lung weight relative to tibia length (mg/mm) in DCM-2Tg and DCM-2Tg×IP₃-R(2)^{-/-} mice. Values shown are mean±SEM (n=11 for the DCM-2Tg group and 14 for the DCM-2Tg × IP₃-R(2)^{-/-} group). **E**, Fractional shortening measured by echocardiography in sham-operated and TAC mice before and 1 and 4 weeks after surgery. Values shown are mean±SEM (n=6 for the sham-operated groups, 13 for +/+ TAC, and 15 for -/- TAC). **F**, LV weight relative to body weight in sham-operated and TAC mice 4 weeks after surgery. Values shown are mean±SEM (n=6 for sham groups, 13 for +/+ TAC, and 15 for -/- TAC). ††P<0.001 relative to sham. There was no significant effect of IP₃-R(2)^{-/-} on any parameter, irrespective of the DCM-2Tg or TAC status, and there was no interaction detected between IP₃-R(2)^{-/-} and DCM-2Tg or TAC. Except for the survival study (**A**), all measurements on DCM-2Tg mice were made between 6 and 8 weeks of age.

Life span also was similar in the 2 groups (Figure 4A), showing that elimination of IP₃-R(2) did not slow disease progression. ECG studies on DCM mice revealed increased P-R interval indicative of conduction block and long Q-T interval, reflecting prolonged action potential duration. Neither of these perturbations was altered by deletion of IP₃-R(2) (Table 3). Hypertrophic responses to pressure overload also were not altered by removal of IP₃-R(2). At either 1 week or 4 weeks after TAC, WT and IP₃-R(2)^{-/-} mice showed similar changes in ventricular dimensions and ventricular weight (Figures 2 and 4F). Failure to detect any effect of deleting IP₃-R(2) on any of the pathological changes observed in either DCM-2Tg mice or mice subjected to TAC argues strongly that Ins(1,4,5)P₃ and its receptors do not make a major contribution, either positively or negatively, to hypertrophy, chamber dilatation, or conduction block in these models.

Heightened IP₃-R expression has been a common finding in a range of cardiac pathologies. Heightened left ventricular expression has been reported in failing human heart,^{1,23} in a rabbit model of nonischemic heart failure,¹⁸ as well as in the murine DCM model used in this study and after TAC (Figure 1). IP₃-R expression/activity is also increased in atrial tissue from patients with atrial fibrillation associated with valvular heart disease.^{9,24} In the latter study, the increased

IP₃-R expression reversed after cardioversion to sinus rhythm, suggesting a contribution of IP₃-R to AF in this patient group. However, the expression of several ion channels and transporters showed a similar reversion with return to sinus rhythm, and the observed changes might be a consequence of, rather than a contributor to AF, as suggested by another study.²⁵ In contrast to IP₃-R expression studies, assessment of changes in the generation of Ins(1,4,5)P₃ in heart disease states has been limited. Our previous studies have shown increased generation of Ins(1,4,5)P₃ in atrial tissue from patients with valve disease, in hypertrophied ventricle from mice after chronic pressure overload, in atria and ventricles of the murine DCM model, and in rodent hearts subjected to acute ischemia/reperfusion.^{4,8,26–28} Other laboratories have also reported heightened Ins(1,4,5)P₃ generation in hearts undergoing hypertrophy²⁹ or after ischemia or ischemia/reperfusion.^{30–32} Thus, heightened generation of Ins(1,4,5)P₃ like increased IP₃-R expression, may be a common feature in cardiac pathologies. It is important to note that production of Ins(1,4,5)P₃ is an indication of phospholipase C activation after receptor activation. Ins(1,4,5)P₃ is generated from the sarcolemmal phospholipid phosphatidylinositol(4,5)-bisphosphate along with *sn*-1,2-diacylglycerol. Thus, increases in Ins(1,4,5)P₃ indicate increased *sn*-1,2-diacylglycerol generation to

activate subtypes of protein kinase C and protein kinase D,^{33,34} as well as some sarcolemmal canonical transient receptor potential channels.³⁵ Any of these *sn*-1,2-diacylglycerol-induced responses could have major influences on disease progression that are independent of IP₃-R. Changes in the precursor phosphatidylinositol(4,5)-bisphosphate also may be of critical significance, because this lipid is a direct regulator of the activity of a range of sarcolemmal ion channels critical in the maintenance of cardiac rhythm.^{35–38} It is also possible that Ins(1,4,5)P₃ has functions other than those that require IP₃-R activation. Ins(1,4,5)P₃ is the precursor of the highly phosphorylated inositol derivatives (InsP4-InsP8),^{39,40} some of which are known to be functionally important,⁴¹ but currently such functions have not been described in heart.

These studies show that IP₃-R(2) do not make a significant contribution to disease in the DCM-2Tg or TAC models, despite the increased activity of the Ins(1,4,5)P₃/IP₃-R(2) axis. We have recently reported that IP₃-R(2) contribute to the regulation of pacemaker function in mice by demonstrating a lowering of the Ca²⁺ responses (by ≈12%) in isolated sinoatrial node tissue in IP₃-R(2)–/– mice.²¹ In the current study, we did not detect any effect of deletion of IP₃-R(2) on heart rate in DCM-2Tg mice, TAC mice, or in WT or sham-operated littermates. However, this contribution of IP₃-R(2) to pacemaker activity would be expected to be buffered by the autonomic nervous system in *in vivo* studies.

In conclusion, we have demonstrated that Ins(1,4,5)P₃ and its receptors do not contribute to disease in murine models of DCM and hypertrophy, both of which have heightened Ins(1,4,5)P₃ generation and increased IP₃-R(2) expression. This finding shows that heightened expression of IP₃-R in heart disease cannot be assumed to have functional significance.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The content of inositol(1,4,5)-trisphosphate (Ins(1,4,5)P₃ [IP₃]) receptors in the heart, IP₃-R(2), has repeatedly been shown to be elevated under pathological conditions, particularly heart failure, valvular heart disease, and atrial fibrillation in human and animal models. Because IP₃-R(2) are regulators of intracellular Ca²⁺, the increased IP₃-R(2) activity has been suggested to contribute to the perturbed Ca²⁺ signaling central to heart diseases. However, there are many factors that change with disease progression, and the challenge is to identify those that contribute to disease, either positively or negatively. In the current study, we provide evidence that IP₃-R(2) do not contribute to heart disease in 2 mouse models, one of dilated cardiomyopathy and the other of pressure overload hypertrophy. This is despite clear elevation in IP₃-R(2) expression, together with increased generation of the ligand, IP₃. Although our data do not rule out a contribution to human disease, it is clear that demonstrating heightened expression or activity of IP₃-R(2) cannot be assumed to influence disease progression. It is likely that factors other than IP₃-R(2) are the source of altered Ca²⁺ signaling in human heart diseases.

SUPPLEMENTAL MATERIAL

Supplementary Methods

Echo cardiography

After a short-axis 2-D image of the LV at the level of the papillary muscles of the LV was obtained, 2-D guided M-mode images were acquired digitally at a sweep speed of 100 mm/s. Images were blindly analyzed as described previously¹. Left ventricular (LV) wall thicknesses [LV posterior wall (LVPW) and interventricular septum (IVS)], LV internal diameter (LVID) at end-diastole and end-systole (LVIDd and LVIDs), and fractional shortening [FS=(LVDd-LVDs)/LVDd] were determined from M-mode images. Left atrial size was determined from long-axis 2-D images at end-systole.

Surface ECG recordings and analyses

ECG recordings were measured in anesthetized mice (isofluorane, 1.7%) using the Powerlab System and BioAmp (ADInstruments). Animals were kept warm using a heating pad (37°C). Two pairs of 27G needle electrodes were placed subcutaneously and recordings were made from a chest lead (equivalent to V5). All signals were sampled at 1 kHz for a period of 5-10 min. Averaged HRs, P-R intervals, R-R intervals, QRS intervals and amplitudes of positive R- and P-waves were measured digitally using ADInstruments (Chart 5 Pro ECG analysis module).

Measurement of mRNA expression.

The following primer sets for mouse were used; IP₃-R(2) (5' AAGGTGTGCCCTATGAATCG, 3' ACCTCTTGTTACCGTCAGG), IP₃-R(1) (5' GCAGAGCCAGAGAAAGAGGA, 3' GTTTCCGGAGGATGTTTCTG), IP₃-R(3) (5' TGATGGACACCAAGCTGAAG, 3' TGATGGTAGCAGTGTGTCTGG) atrial natriuretic peptide (ANP) (5' ATCTGCCCTATTGAAAAGCA, 3' ACACACCACAAGGGCTTAGG), α -skeletal actin (5' CCGACCCCGTCACCAGGGTG, 3' ATCCAACACGATGCCGGTG), MCIP, (5' TCCAGCTTGGGCTTGACTGAG, 3' ACTG GAAGGTGGTGTCTTGTC) α -MHC (5' CTGCTGGAGAGGTTATTCCTCG, 3' GGAAGAGTGAGCGGCGCATCAAGG), β -MHC (5'

TGCAAAGGCTCCAGGTCTGAGGGC 3' GCCAACACCAACCTGTCCAAGTTC) Mouse GAPDH (5' ATGACTCCACTCACGGCAAAT, 3' TCCCATTCTCGGCCTTGAC).

Western blot analysis

Total protein was extracted from left ventricles of IP₃-R(2)^{+/+} and IP₃-R(2)^{-/-} mice. A polyclonal antibody to IP₃R(2) was produced in rabbits immunized with a synthetic peptide for mouse IP₃R(2) (amino acid sequence, RKNKQRLGFLGSNTPH; Open Biosystems, 1:500), and was used for immunodetection as previously described ².

Measurement of Ins(1,4,5)P₃ generation

Atrial and ventricular tissues were labeled with [³H]inositol and subsequently stimulated with norepinephrine (50 μmol/L), in the presence of 1 μM propranolol and 10 mM LiCl for 20 min. Atria were labelled with [³H]inositol and subsequently stimulated in 96 well tissue culture dishes. Ventricles were perfused with medium containing [³H]inositol by the Langendorf method, and data include both left and right ventricle. [³H]-Labeled Ins(1,4,5)P₃ and its metabolites were extracted and subsequently quantified by HPLC, as described previously ^{3 4}.

Supplementary data

IP₃-R(1) and IP₃-R(3) expression in LV for WT and DCM-2Tg mice with and without IP₃-R(2).

IP₃-R(2) are thought to be exclusively expressed in working cardiomyocytes, IP₃-R(1) are regarded the sole subtype expressed in the conducting tissue, but expression on IP₃-R(3) has not been reported in cardiomyocytes. The possible effect of deletion of IP₃-R(2) on the expression levels of IP₃-R(1) and IP₃-R(3) in heart was assessed. There was no significant effect of deletion of IP₃-R(2) on the expression of IP₃-R(1) or IP₃-R(3) in any chambers of the hearts (Supplemental Figure 1). As expected for a subtype not expressed in cardiomyocytes, expression of IP₃-R(3) was extremely low, approximately 1/10 of the other two subtypes. There was no alteration in the expression of IP₃-R(3) caused by deletion of IP₃-R(2).

Deletion of IP₃-R(2) did not alter the pressure gradient induced by TAC.

As shown in Supplemental Figure 2A, there was no significant difference in trans-stenotic pressure gradients between IP₃-R(2)^{+/+} (WT) and IP₃-R(2)^{-/-} mice.

Deletion of IP₃-R(2) did not alter echocardiographic parameters or ECG data in DCM-2Tg mice or in littermate controls (WT).

As shown in Supplemental Figure 2B echo cardiographic profiles in WT or DCM-2Tg mice were not altered by deletion of IP₃-R(2). Similarly, ECG recordings were similar either in WT or DCM-2Tg with or without IP₃-R(2) (Supplemental Figure 2C).

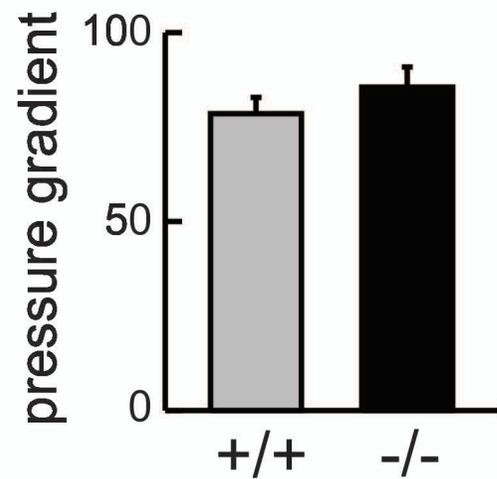
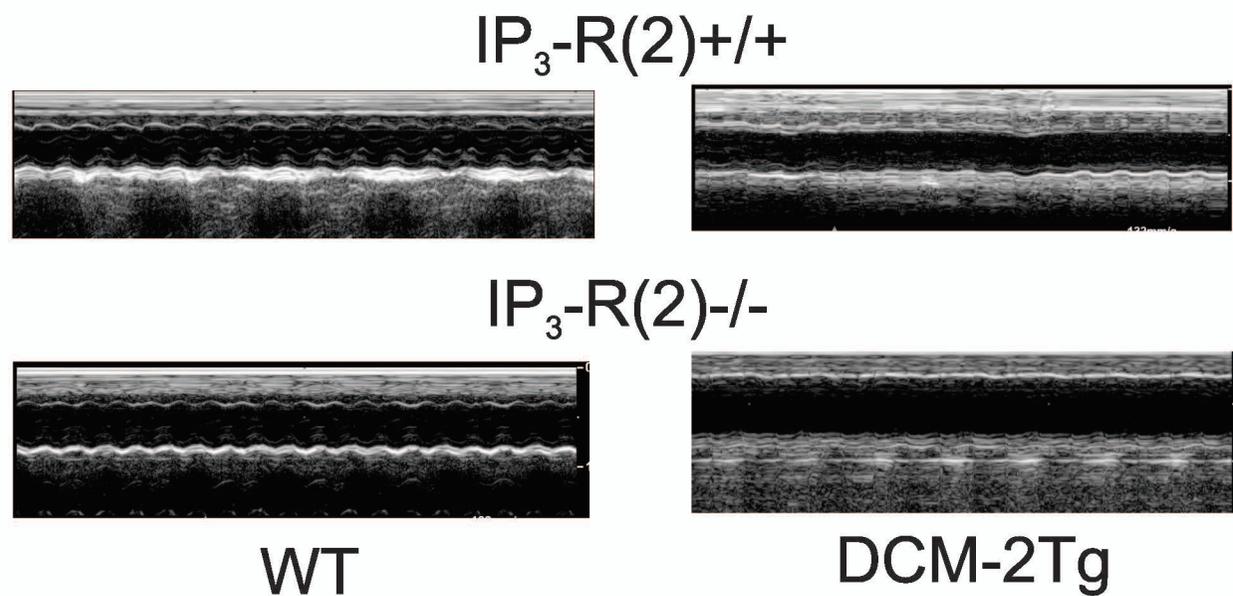
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Supplemental Figure Legends

Supplemental Figure 1. *Lack of effect of deletion of IP₃-R(2).* **A.** Transstenotic pressure gradient measured in IP₃-R(2) +/+ and IP₃-R(2) -/- mice. **B.** Representative echocardiographic profiles in WT and DCM-2Tg mice with and without IP₃-R(2). **C.** Representative ECG profiles in WT and DCM mice with and without IP₃-R(2).

Supplemental Figure 2. *Expression of IP₃-R(1) and IP₃-R(3) in left ventricle of mice with and without their complement of IP₃-R(2).* Values shown are mRNA expression relative to GAPDH, mean ± sem, n=4-8. No values differed between the relevant IP₃-R(2)/+/+ and IP₃-R(2)/-/- groups (1 way ANOVA).

A**B****C**