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

Cooley et al: No Effect of IP₃-R(2) on DCM or Hypertrophy

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Abstract

**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 x dn-PI3K-2Tg; DCM-2Tg) develop severe DCM and conduction block, associated with increased expression of 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 thoracic aortic constriction (TAC).

**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 x IP3-R(2)-/-) and TAC was performed on IP3-R(2)-/- mice. Hearts from DCM-2Tg mice and DCM-2Tg x 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, as DCM-2Tg mice with and without IP3-R(2) had similarly reduced contractility, increased lung congestion, atrial thrombus and both strains died between 10 and 12 weeks of age. Loss of IP3-R(2) did not alter the progression of hypertension following TAC.

**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.

**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 Ca\textsuperscript{2+}-regulating second messenger Ins(1,4,5)P\textsubscript{3} (IP\textsubscript{3}-receptors, IP\textsubscript{3}-R), often along with a decrease in ryanodine receptors, the master regulators of Ca\textsuperscript{2+} within the myocardium\textsuperscript{1}. The importance, or otherwise, of this has been debated. The altered relative expression levels of the intracellular Ca\textsuperscript{2+} channels might reflect a degree of de-differentiation of the failing ventricular myocytes, with no major functional implications. Alternatively, increased IP\textsubscript{3}-R might contribute to, or ameliorate, the progression of heart failure. IP\textsubscript{3}-R have been suggested to contribute to cardiomyocyte hypertrophy\textsuperscript{2} \textsuperscript{3}, to chamber dilatation\textsuperscript{4} and to arrhythmogenesis\textsuperscript{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)P\textsubscript{3} in response to the activation of appropriately coupled receptors\textsuperscript{6}, and in addition the expression of IP\textsubscript{3}-R is low\textsuperscript{7}. These findings argue against a strong involvement of IP\textsubscript{3}-R under physiological conditions. However, our earlier and recent studies show that generation of Ins(1,4,5)P\textsubscript{3} is markedly heightened under such pathological conditions as atrial dilatation in humans, mice with dilated cardiomyopathy\textsuperscript{4} and in hypertrophied mouse ventricle following chronic pressure overload\textsuperscript{8}. Furthermore, we have observed heightened expression of phospholipase C\textbeta\textsubscript{1b} (PLC\textbeta\textsubscript{1b}), the enzyme responsible for generation of Ins(1,4,5)P\textsubscript{3} in myocytes, from failing human, mouse and sheep myocardium\textsuperscript{4} (sheep data not shown). All of these findings suggest that Ins(1,4,5)P\textsubscript{3} may be of heightened importance in failing myocardium, especially given the likelihood of increased IP\textsubscript{3}-R expression\textsuperscript{7} \textsuperscript{9}. 
We undertook an investigation of possible pathological roles of IP$_3$-R using a mouse model with severe dilated cardiomyopathy (DCM) and clear evidence of conduction block (DCM-2Tg) as well as a well characterized model of pressure overload hypertrophy, induced by trans-aortic constriction (TAC) $^8$. Both of these murine model show increased generation of Ins(1,4,5)P$_3$ $^4$ $^8$ and increased expression of IP$_3$-R(2), the IP$_3$-R subtype expressed in working cardiomyocytes. To examine possible contributions of the heightened Ins(1,4,5)P$_3$ and IP$_3$-R(2) to hypertrophy and dilatation, we performed TAC on IP$_3$-R(2)/-/- mice and crossed the DCM-2Tg with IP$_3$-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 $^{10}$ $^{11}$. IP$_3$-R(2)/-/- mice were generated on a C57BL6 background, as described $^5$. Male heterozygous Mst1-Tg (Mst1$^{+/-}$) were genetically crossed with dnPI3K$^{+/+}$ and IP$_3$-R(2)/-/- to generate DCM-2Tg mice (Mst1$^{+/-}$, Mst1xdnPI3K-2Tg, DCM-2Tg) and DCM-2Tg-KO mice (Mst1$^{+/-}$/dnPI3K$^{+/+}$/IP$_3$-R(2)/-/-) $^{12}$. The crossed strain (Mst1xdnPI3K2-Tg) was chosen for these studies because, unlike the Mst1-Tg, they show severe conduction block and we were interested in possible atrial actions of IP$_3$-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
thoracic aortic constriction (TAC) as previously described. Echocardiography measurements were performed before surgery, 1 and 4 weeks post-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 WT littermates were similar (Supplemental Figure 1A). Hearts were collected and morphometrics evaluated 4 weeks post-TAC.

**Transthoracic echocardiography and electrocardiography (ECG)**

Anesthesia was maintained with 1.7% isoflurane. Echocardiography was performed using a Philips iE33 ultrasound machine and a 15 MHz linear-transducer. 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 supplement.

**Measurement of Ins(1,4,5)P₃ and its metabolites in atria and ventricles (PLC 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 HPLC, as described previously. Details are provided in the online 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 qRT-PCR with SybrGreen (Invitrogen) reagent was performed on an Applied Biosciences 7500 Fast Real Time PCR System. Primer sequences are provided in the online supplement. Values are expressed as the 2⁻ΔCt value relative to GAPDH in each sample. All qRT-PCR experiments were performed in triplicate on triplicate or quadruplicate samples.
RNA dot-blot analyses were performed as previously described \(^{16}\). Protein methods are outlines in the online supplement.

**Data evaluation**

For experiments involving two 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, IP\(_3\)-R(2)-/-, DCM, DCM x IP\(_3\)-R(2)-/- or Sham, sh. IP\(_3\)-R(2)-/-, TAC, TAC IP\(_3\)-R(2)-/-) a 2-Way ANOVA was used to assess the effect of TAC or DCM, the IP\(_3\)-R(2) status and the interaction between the two (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 test post hoc, 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 IP\(_3\)-R(2) and heightened generation of Ins(1,4,5)P\(_3\)*

The type 2 IP\(_3\)-R is expressed in working cardiomyocytes and we measured expression in hearts from DCM-2Tg mice that have dilated cardiomyopathy as well as from mice that have undergone TAC to induce pressure overload hypertrophy. The DCM-2Tg mice had heightened in IP\(_3\)-R(2) expression in atria and left ventricles (Figure 1A). Loss of IP\(_3\)-R(2)in IP\(_3\)-R(2)-/-mice, did not result in altered expression of either of the other IP\(_3\)-R subtypes, IP\(_3\)-R(1) or IP\(_3\)-R(3) (Supplemental Figure 1). IP\(_3\)-R(2) expression also was substantially elevated 4 weeks after TAC (Figure 1B&C).
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)P$_3$ \(^8\). In the current study we assessed Ins(1,4,5)P$_3$ generation in atria and ventricles from DCM-2Tg and littermate WT mice. This was accomplished by labeling the inositol phospholipids with \( ^{3}H \)inositol and subsequently measuring the generation of \( ^{3}H \)-labeled Ins(1,4,5)P$_3$ and its metabolites as a measure of total \( ^{3}H \)Ins(1,4,5)P$_3$ generation. As described previously \(^4\), atria and ventricles from WT mice have low levels of \( ^{3}H \)InsPs after 20 min treatment with norepinephrine (50 \( \mu \)M), indicating minimal generation of Ins(1,4,5)P$_3$. Responses were substantially heightened in atria and ventricles from DCM-2Tg mice reflecting increased Ins(1,4,5)P$_3$ production (Figure 1D).

**Deletion of IP$_3$-R(2) does not influence heart size in DCM-2Tg mice or TAC mice.**

DCM-2Tg mice develop dilated cardiomyopathy indicated by chamber dilatation, LV wall thinning and decreased contractile function \(^12\). We argued that heightened generation of Ins(1,4,5)P$_3$, together with heightened expression of IP$_3$-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 IP$_3$-R(2) had been deleted (DCM-2TgxIP$_3$-R(2)-/-), and compared this with DCM mice expressing the endogenous complement of IP$_3$-R(2) (DCM-2Tg). As reported previously \(^12\), DCM mice exhibited severe LV dilatation (Supplemental Figure 2B, 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 IP$_3$-R(2) deleted showed a phenotype indistinguishable from that of DCM-2Tg mice (Table 1, 2).

In contrast to the DCM-2Tg model, mice subjected to TAC develop ventricular hypertrophy indicated by increased LV/body weight ratio and increased ventricular wall thickness. Deletion of IP$_3$-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 were 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 (ANP) and α-skeletal actin (α-Sk. actin). Deletion of IP3-R(2) did not alter these expression changes. As reported previously 8, TAC induced changes in ‘hypertrophic’ gene expression profiles, ANP, α-myosin heavy chain (MHC), β-MHC and α-skeletal actin, and these were not altered by deletion of IP3-R(2) (Figure 3C&D).

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 NFAT 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 (MCIP) is a direct transcriptional target of calcineurin and we measured MCIP as an index of calcineurin signaling. As shown in Figure 3B, MCIP expression was increased at 4 weeks following TAC, but deletion of IP3-R(2) did not influence expression either in TAC or 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 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 IP$_3$-R(2) did not influence these changes and data obtained from DCM-2Tg and DCM-2TgxIP$_3$-R(2)-/- were not different in terms of any of these parameters. Deletion of the IP$_3$-R(2) also did not alter ECG profiles in the absence of DCM (Supplemental Figure 2C, Table 3).

**Deletion of IP$_3$-R(2) does not alter functional parameters in DCM-2Tg mice or in TAC mice.**

DCM-2Tg mice die prematurely by 10-12 weeks of age. Deletion of IP$_3$-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 IP$_3$-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 IP$_3$-R(2) (Figure 4C, Table 1). Lung congestion was similar in DCM-2Tg with and without their complement of IP$_3$-R(2) (Figure 4D). Thus, IP$_3$-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 IP$_3$-R(2) deletion (Figure 4E). Similarly increases in left ventricular weight were similar in WT and IP$_3$-R(2)-/- mice following TAC (Figure 4F).

**Discussion**

Heart failure in humans and experimental animals has repeatedly been reported to be associated with increased ventricular expression of IP$_3$-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 IP$_3$-R(2) expression has been reported in neonatal rat cardiomyocytes undergoing
hypertrophy. In some cases the increased IP$_3$-R(2) expression is paralleled by a lowering of ryanodine receptor expression. These findings might suggest that IP$_3$-R(2) are of increased importance in hypertrophic and failing myocardium. It has been suggested that the increased IP$_3$-R activity contributes to pathology by promoting hypertrophic growth of the cardiomyocytes or that the increased IP$_3$-R might contribute to arrhythmia by perturbing Ca$^{2+}$ responses close to sarcolemmal Ca$^{2+}$ channels and/or the Na$^+$/Ca$^{2+}$ exchanger. Alternatively, the changes might reflect a loss of muscle phenotype as 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)P$_3$, 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.

Transgenic mice expressing Mst1 in heart have been reported previously to have dilated cardiomyopathy, and the phenotype is exacerbated by co-expression of dominant negative PI3-Kinase. The crossed strain (Mst1xdn-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 interval (Table 3). Both the Mst1-Tg and Mst1xdn-PI3K-2Tg strains express heightened levels of IP$_3$-R(2) in atria and ventricles and additionally both have substantially increased Ins(1,4,5)P$_3$ generation (Figure 1). We reasoned that the heightened Ins(1,4,5)P$_3$/IP$_3$-R axis in this mouse strain provided an excellent opportunity to evaluate its contribution to the DCM phenotype. The crossed strain (Mst1xdn-PI3K-2Tg; DCM-2Tg) was chosen for these studies because we were interested in a possible contribution of Ins(1,4,5)P$_3$ and IP$_3$-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. In addition to a contribution to heart failure, IP3-R have been suggested to contribute to hypertrophic signaling pathways, by supplying Ca\(^{2+}\) to activate the calcineurin/NFAT pathway, or by activating CaMKII localized close to the nuclear membrane. To evaluate possible contributions of the Ins(1,4,5)P\(_3\)/IP3-R(2) axis to hypertrophy we used a well-established pressure overload model.

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 two experimental groups. Life span also was similar in the two 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 (Figure 2, 4F). Failure to detect any effect of deleting IP3-R(2) on any of the pathological changes observed in either DCM-2Tg mice or in mice subjected to TAC argues strongly that Ins(1,4,5)P\(_3\) 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, in a rabbit model of non-ischemic heart failure as well as in the murine DCM model used in
this study and following TAC (Figure 1). IP$_3$-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 IP$_3$-R expression reversed following cardioversion to sinus rhythm, suggesting a contribution of IP$_3$-R to AF in this patient group. However, the expression of a number of 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 IP$_3$-R expression studies, assessment of changes in the generation of Ins(1,4,5)P$_3$ in heart disease states has been limited. Our previous studies have shown increased generation of Ins(1,4,5)P$_3$ in atrial tissue from patients with valve disease, in hypertrophied ventricle from mice following 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)P$_3$ generation in hearts undergoing hypertrophy $^{29}$ or following ischemia or ischemia/reperfusion $^{30-32}$. Thus, heightened generation of Ins(1,4,5)P$_3$, like increased IP$_3$-R expression, may be a common feature in cardiac pathologies. It is important to note that production of Ins(1,4,5)P$_3$ is an indication of phospholipase C activation following receptor activation. Ins(1,4,5)P$_3$ is generated from the sarcolemmal phospholipid phosphatidylinositol(4,5)bisphosphate (PIP$_2$) along with sn-1,2-diacylglycerol (DAG). Thus, increases in Ins(1,4,5)P$_3$ indicate increased DAG generation to activate subtypes of protein kinase C and protein kinase D $^{33,34}$, as well as some sarcolemmal canonical transient receptor potential (TrpC) channels $^{35}$. Any of these DAG-induced responses could have major influences on disease progression that are independent of IP$_3$-R. Changes in the precursor PIP$_2$ also may be of critical significance, as 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 important⁴¹, 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 approximately 12%) in isolated sinoatrial node tissue in IP₃-R(2)-/- mice²¹. 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 a murine models of DCM and of 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.
Acknowledgements

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Disclosures

None.

References


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Table 1

*Echocardiographic parameters in DCM-2Tg mice and the effect of deletion of IP₃-R(2)*

<table>
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<th></th>
<th>WT (7)</th>
<th>IP₃-R(2)-/- (5)</th>
<th>DCM-2Tg (6)</th>
<th>DCM-2Tg x IP₃-R(2)-/- (12)</th>
<th>p DCM</th>
<th>p IP₃-R(2)-/-</th>
<th>p int.</th>
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</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>males/females</td>
<td>5/2</td>
<td>4/1</td>
<td>2/4</td>
<td>5/7</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (weeks)</td>
<td>9.1 ± 1.4</td>
<td>7.2 ± 0.5</td>
<td>8.0 ± 0.1</td>
<td>8.8 ± 0.7</td>
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<tr>
<td>HR, bpm (echo)</td>
<td>571 ± 30</td>
<td>628 ± 48</td>
<td>490 ± 34</td>
<td>507 ± 20</td>
<td>ns</td>
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<td>ns</td>
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<td>LVPWs mm</td>
<td>1.3 ± 0.10</td>
<td>1.2 ± 0.05</td>
<td>0.75 ± 0.05</td>
<td>0.8 ± 0.05</td>
<td>&lt;0.001</td>
<td>0.619</td>
<td>0.943</td>
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<tr>
<td>LVPWd mm</td>
<td>0.92 ± 0.09</td>
<td>0.94 ± 0.06</td>
<td>0.66 ± 0.04</td>
<td>0.68 ± 0.05</td>
<td>&lt;0.001</td>
<td>0.516</td>
<td>0.201</td>
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<td>LVIDs mm</td>
<td>1.97 ± 0.18</td>
<td>2.2 ± 0.26</td>
<td>3.69 ± 0.14</td>
<td>3.7 ± 0.18</td>
<td>&lt;0.001</td>
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<tr>
<td>LVIDd mm</td>
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<td>4.2 ± 0.18</td>
<td>4.3 ± 0.16</td>
<td>&lt;0.001</td>
<td>0.567</td>
<td>0.660</td>
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<tr>
<td>IVSs mm</td>
<td>1.53 ± 0.11</td>
<td>1.4 ± 0.12</td>
<td>0.79 ± 0.06</td>
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<tr>
<td>IVSd mm</td>
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<td>0.71 ± 0.04</td>
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<tr>
<td>LA area mm²</td>
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<td>7.24 ± 0.9</td>
<td>21.7 ± 2.3</td>
<td>19.68 ± 1.6</td>
<td>&lt;0.001</td>
<td>0.593</td>
<td>0.637</td>
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</table>

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)-/-. ns = not significant.
Table 2

*Morphometric parameters in DCM-2Tg mice and the effect of deletion of IP3-R(2)*

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>IP3-(2)/-</th>
<th>DCM-2Tg</th>
<th>DCM-2Tg x IP3-R(2)/-</th>
<th>p DCM</th>
<th>p IP3-R(2)/-</th>
<th>p Int.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m/f)</td>
<td>4/2</td>
<td>4/2</td>
<td>5/7</td>
<td>8/10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, weeks</td>
<td>13 ± 0.58</td>
<td>10 ± 0.75</td>
<td>10.5 ± 1.3</td>
<td>11.8 ± 1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventricle/tibia, mg/mm</td>
<td>7.05 ± 0.17</td>
<td>6.85 ± 0.41</td>
<td>6.41 ± 0.41</td>
<td>6.47 ± 0.30</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Atria/tibia, mg/mm</td>
<td>0.39 ± 0.09</td>
<td>0.37 ± 0.05</td>
<td>0.94 ± 0.2</td>
<td>0.88 ± 0.11</td>
<td>&lt;0.001</td>
<td>0.486</td>
<td>0.294</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>28.2 ± 2.4</td>
<td>31.4 ± 3.8</td>
<td>23.8 ± 1.6</td>
<td>26.8 ± 1.3</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

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)/-. ns = not significant.
Table 3

**ECG parameters in DCM-2Tg mice and the effect of deletion of IP$_3$-R(2)**

<table>
<thead>
<tr>
<th></th>
<th>WT (8)</th>
<th>IP$_3$-R(2)-/- (5)</th>
<th>DCM-2Tg (8)</th>
<th>DCM-2Tg x IP$_3$-R(2)-/- (13)</th>
<th>p DCM</th>
<th>p IP$_3$-R(2)-/-</th>
<th>p Int.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m/f)</td>
<td>5/3</td>
<td>3/2</td>
<td>4/4</td>
<td>5/8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>9.3 ± 1</td>
<td>10 ± 3</td>
<td>9.2 ± 0.8</td>
<td>11.4 ± 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R amp., mV</td>
<td>1.26 ± 0.2</td>
<td>1.15 ± 0.16</td>
<td>0.66 ± 0.09</td>
<td>0.86 ± 0.11</td>
<td>&lt;0.001</td>
<td>0.252</td>
<td>0.950</td>
</tr>
<tr>
<td>P amp, mV</td>
<td>0.13 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.057 ± 0.01</td>
<td>0.048 ± 0.01</td>
<td>&lt;0.001</td>
<td>0.374</td>
<td>0.500</td>
</tr>
<tr>
<td>P-R int. ms</td>
<td>37 ± 1</td>
<td>35 ± 2</td>
<td>54 ± 4</td>
<td>56 ± 3</td>
<td>&lt;0.001</td>
<td>0.825</td>
<td>0.485</td>
</tr>
<tr>
<td>Q-T int. ms</td>
<td>17 ± 1.5</td>
<td>14 ± 1</td>
<td>42 ± 5</td>
<td>39 ± 3</td>
<td>&lt;0.001</td>
<td>0.482</td>
<td>0.239</td>
</tr>
<tr>
<td>QRS int. ms</td>
<td>8 ± 1</td>
<td>8 ± 1</td>
<td>10 ± 1</td>
<td>10 ± 1</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>R-R int. ms</td>
<td>112 ± 7</td>
<td>111 ± 4</td>
<td>119 ± 5</td>
<td>124 ± 2</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

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 IP$_3$-R(2)-/- and the interaction (int.) between DCM and IP$_3$-R(2)-/-. ns = not significant.
Figure Legends

**Figure 1. Hearts of DCM-2Tg mice and TAC mice have heightened IP₃-R(2) expression and InsP generation.**  
A. Expression of IP₃-R(2) in atria and ventricles from DCM-2Tg mice and littermate controls (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 times 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, CPM/mg of tissue, mean ± SEM, n=5-7, * p<0.05 relative to WT or sham operated control (Mann-Whitney, Rank Sum Test).

**Figure 2. Deletion of IP₃-R(2) does not alter hypertrophic responses at 1 or 4 weeks after TAC.**  
Echocardiographic parameters measured in IP₃-R(2)-/- mice and littermate controls (WT, +/-), pre-TAC and at 1 week 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, † p<0.05. There was no significant effect of IP₃-R(2)-/- on any parameter irrespective of the TAC status.

**Figure 3. Lack of effect of IP₃-R(2)-/- on transcriptional changes in DCM-2Tg mice or TAC mice.**  
A. Expression of hypertrophic marker genes in left ventricle from the DCM-2Tg and 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 LV 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, † 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.

**Figure 4.** Deletion of IP3-R(2) does not alter the phenotype of the DCM-2Tg mice or TAC mice. **A.** Kaplan-Meier survival curves of DCM-2Tg mice, 10 total, (black symbols) and DCM-2Tg x IP3-R(2)-/- mice, 10 total (red symbols). There was no detectable difference between death rates. **B.** Incidence of pleural effusion, LA and 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 7/13 females and the -/- group 10/19 females. None of the values was significantly different between the two groups. **C.** Fractional shortening, measured by echocardiography, in DCM-2Tg mice and DCM-2Tg x IP3-R(2)-/- mice. Values shown are mean ± SEM (n=6 for the DCM-2Tg group and 12 for the DCM-2Tg x IP3-R(2)-/- group). †† p<0.001 relative to WT or IP3-R(2)-/- mice. **D.** Lung wt. relative to tibia length (mg/mm) in DCM-2Tg and DCM-2Tg x IP3-R(2)-/- mice. Values shown are mean ± SEM (n=11 for the DCM-2Tg group and 14 for the DCM-2Tg x 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 IP$_3$-R(2)-/- on any parameter irrespective of the DCM-2Tg or TAC status and there was no interaction detected between IP$_3$-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.
Fig. 2
Fig. 3

A. ANP and α-Sk. actin levels in WT and DCM mice.

B. MCIP mRNA/GAPDH levels in Sh. and TAC groups.

C. Western blot analysis of ANP, α-MHC, β-MHC, α-Sk. actin, and GAPDH in +/+ and -/- WT and DCM mice.

D. mRNA/GAPDH levels of α-MHC and β-MHC in Sh. and TAC groups.
No Contribution of IP₃-R(2) to Disease Phenotype in Models of Dilated Cardiomyopathy or Pressure Overload Hypertrophy
Nicola Cooley, Küngfu Ouyang, Julie R. McMullen, Helen Kiriazis, Farah Sheikh, Wei Wu, Yongxin Mu, Xiao-Jun Du, Ju Chen and Elizabeth A. Woodcock

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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 previously 1. 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’ AAGGTGTGCCCCTATGAATCG, 3’ Accttgttctacggcgtcagg), IP3-R(1) (5’ GCAGAGCCAGAAGAGAGGA, 3’ Gtttcggaggatgtttctg), IP3-R(3) (5’ TGATGGACACCAAGCTGAAG, 3’ Tgatggtagcagtgtgtctgg) atrial natriuretic peptide (ANP) (5’ ATCTGCCCTATTGAAAGCA, 3’ ACACACCACAAGGGCTTAGG), α-skeletal actin (5’ CCGACCCCCTCACCAGGCTGTG, 3’ ATCCAACACGATGCCGGTG), MCIP, (5’ CCGACCCCCTCACCAGGCTGTG, 3’ ATCCAACACGATGCCGGTG), α-MHC (5’ CTGCTGGAGGTTATTCTCG, 3’ GGAAGAGTGAGCCGCATCAAAG), β-MHC (5’
TGCAAAGGCTCCAGGTCTGAGGGG 3’ GCCAACACCAACCTGTCCAAGTTC) Mouse GAPDH (5’ ATGACTCCACTCAGGCAAAT, 3’ TCCCATTCTCGGCCTTGAC).

**Western blot analysis**

Total protein was extracted from left ventricles of IP$_3$-R(2)+/+ and IP$_3$-R(2)-/- mice. A polyclonal antibody to IP$_3$R(2) was produced in rabbits immunized with a synthetic peptide for mouse IP$_3$.R(2) (amino acid sequence, RKNKQLRGLFSNTPH; Open Biosystems, 1:500), and was used for immunodetection as previously described.

**Measurement of Ins(1,4,5)P$_3$ generation**

Atrial and ventricular tissues were labeled with [${}^3$H]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 [${}^3$H]inositol and subsequently stimulated in 96 well tissue culture dishes. Ventricles were perfused with medium containing [${}^3$H]inositol by the Langendorf method, and data include both left and right ventricle. [${}^3$H]-Labeled Ins(1,4,5)P$_3$ and its metabolites were extracted and subsequently quantified by HPLC, as described previously.

**Supplementary data**

**IP$_3$-R(1) and IP$_3$-R(3) expression in LV for WT and DCM-2Tg mice with and without IP$_3$-R(2).**

IP$_3$-R(2) are thought to be exclusively expressed in working cardiomyocytes, IP$_3$-R(1) are regarded the sole subtype expressed in the conducting tissue, but expression on IP$_3$-R(3) has not been reported in cardiomyocytes. The possible effect of deletion of IP$_3$-R(2) on the expression levels of IP$_3$-R(1) and IP$_3$-R(3) in heart was assessed. There was no significant effect of deletion of IP$_3$-R(2) on the expression of IP$_3$-R(1) or IP$_3$-R(3) in any chambers of the hearts (Supplemental Figure 1). As expected for a subtype not expressed in cardiomyocytes, expression of IP$_3$-R(3) was extremely low, approximately 1/10 of the other two subtypes. There was no alteration in the expression of IP$_3$-R(3) caused by deletion of IP$_3$-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).

References
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).
Suppl. Fig. 1
Suppl. Fig. 2