The IP₃ Receptor Regulates Cardiac Hypertrophy in Response to Select Stimuli

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**Rationale:** Inositol 1,4,5-trisphosphate (IP₃) is a second messenger that regulates intracellular Ca²⁺ release through IP₃ receptors located in the sarcoplasmic reticulum of cardiac myocytes. Many prohypertrophic G protein–coupled receptor (GPCR) signaling events lead to IP₃ liberation, although its importance in transducing the hypertrophic response has not been established in vivo.

**Objective:** Here, we generated conditional, heart-specific transgenic mice with both gain- and loss-of-function for IP₃ receptor signaling to examine its hypertrophic growth effects following pathological and physiological stimulation.

**Methods and Results:** Overexpression of the mouse type-2 IP₃ receptor (IP₃R2) in the heart generated mild baseline cardiac hypertrophy at 3 months of age. Isolated myocytes from overexpressing lines showed increased Ca²⁺ transients and arrhythmias in response to endothelin-1 stimulation. Although low levels of IP₃R2 overexpression failed to augment/synergize cardiac hypertrophy following 2 weeks of pressure-overload stimulation, such levels did enhance hypertrophy following 2 weeks of isoproterenol infusion, in response to Gq overexpression, and/or in response to exercise stimulation. To inhibit IP₃ signaling in vivo, we generated transgenic mice expressing an IP₃ chelating protein (IP₃-sponge). IP₃-sponge transgenic mice abrogated cardiac hypertrophy in response to isoproterenol and angiotensin II infusion but not pressure-overload stimulation. Mechanistically, IP₃R2-enhanced cardiac hypertrophy following isoproterenol infusion was significantly reduced in the calcineurin-AΔF–null background.

**Conclusion:** These results indicate that IP₃-mediated Ca²⁺ release plays a central role in regulating cardiac hypertrophy downstream of GPCR signaling, in part, through a calcineurin-dependent mechanism. (Circ Res. 2010;107:659-666.)

**Key Words:** hypertrophy ■ calcium ■ signaling ■ calcineurin

Cardiac hypertrophy occurs as an adaptive response to various cardiovascular diseases such as hypertension, valvular insufficiency, ischemic heart disease, infectious agents, or mutations in sarcomeric genes.¹ Ca²⁺ underlies excitation–contraction coupling (ECC), and it serves as a second messenger to induce cardiac hypertrophy, in part, by activating select Ca²⁺-dependent reactive signaling proteins such as calcineurin, calmodulin-dependent kinase (CaMK)II, and protein kinase C.²³ However, it remains unknown how Ca²⁺ activates these hypertrophic signaling effectors in the heart given ECC-mediated Ca²⁺ fluxing that bathes the entire cytoplasm of a cardiac myocyte.⁴ One possibility is that specialized pools of Ca²⁺ have evolved that are temporally and spatially distinct from the cytoplasmic Ca²⁺ transient in ECC. For example, CaMKII is activated in cardiomyocytes by a perinuclear Ca²⁺ pool associated with the inositol 1,4,5 trisphosphate (IP₃) receptor.⁵ IP₃ is a second messenger generated by hydrolysis of membrane lipid phosphatidylinositol 4,5-bisphosphate by phospholipase (PL)C in response to GPCR activation associated with growth factors and neuroendocrine agonists.⁶ Once generated, IP₃ causes Ca²⁺ release from intracellular stores by binding the IP₃ receptor (IP₃R), an intracellular Ca²⁺ release channel embedded in the sarcoplasmic reticulum (SR) and nuclear envelope. Cardiac hypertrophy has been associated with increased PLC activity and increased generation of IP₃.⁷⁸ Moreover, expression of IP₃Rs is increased in both human and animal models of heart failure, suggesting that this form of Ca²⁺ release may be associated with pathology.⁹¹⁰

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The IP$_3$R family consists of 3 genes: IP$_3$R1, IP$_3$R2, and IP$_3$R3. IP$_3$R2 is thought to be the most prominent gene expressed in the heart, and its deletion in gene-targeted mice abolished positive inotropy and spontaneous Ca$^{2+}$ release in atrial myocytes caused by endothelin (ET)-1 stimulation. Even though ventricular myocytes express much lower levels of IP$_3$Rs than atrial myocytes, these receptors, in some reports, can alter Ca$^{2+}$ release and predispose to arrhythmia. Although the IP$_3$Rs can affect Ca$^{2+}$ release, it has not been possible to determine their necessity in regulating the cardiac hypertrophic response because all 3 receptor genes are expressed in the heart, complicating a gene-targeting approach, not withstanding lethality issues in IP$_3$R1-null mice. Here, we generated transgenic mice with IP$_3$R2 overexpression and the inhibitory IP$_3$-sponge protein, demonstrating for the first time that the IP$_3$R functions as a hypertrophic effector in vivo.

### Methods

#### Generation of Transgenic Mice

cDNAs encoding mouse IP$_3$R2 and recombinant Flag-tagged IP$_3$-sponge protein were cloned into the murine inducible α-myosin heavy chain (α-MHC) promoter expression vector (gift from Dr Jeffrey Robbins, Children’s Hospital, Cincinnati, Ohio). Geq transgenic mice were kindly provided by Dr Gerald Dorn II. NFAT-luciferase reporter transgenic mice and calcineurin Aβ-null mice (CnAβ$^{-/-}$) were reported previously. To reduce strain effects the CnAβ-null mice (C57BL/6) were backcrossed 6 generations into the IP$_3$R2 (FVB/N) background. Mice were given doxycycline (Sigma, St Louis, Mo) at 1 g/L in the drinking water for 3 weeks to shut down protein expression from the inducible transgenes. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center.

#### Western Blot Analysis

Western blot analysis for mouse ventricle homogenates were performed as previously reported. Antibodies included IP$_3$R2, Gaq, and α-tubulin (Santa Cruz Biotechnology), GAPDH (Research Diagnostics Inc, Flanders, NJ), and Flag M2 monoclonal (Sigma). Chemiluminescent detection was performed with the Vistra ECF reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and scanned with a Storm 860 PhosphorImager (Molecular Dynamics, Piscataway, NJ).

### RT-PCR, Luciferase Assay, Immunohistochemistry, and Histological Analysis

RT-PCR was performed as described previously. For histological analysis, hearts were collected at the indicated times, fixed in 10% formalin containing PBS, and embedded in paraffin. Myocyte cross-sectional areas were analyzed in slides stained with wheat germ agglutinin–FITC. Immunohistochemistry on adult myocytes was performed as described previously using an IP$_3$R2 antibody (Abcam) and α-actinin (Sigma).

### Echocardiography, Invasive Hemodynamics, and Surgical Models

Mice were anesthetized with isoflurane, and echocardiography was performed using a Hewlett Packard 5500 instrument with a 15-MHz microprobe as described previously. Invasive hemodynamics in the closed-chest mouse with a 1.4F Millar catheter was performed as described previously. Transverse aortic constriction (TAC) to induce cardiac pressure overload hypertrophy was described previously. Pressure gradients across the constriction were measured by Doppler echocardiography as described previously. Alzet miniosmotic pumps (no. 2002; Alza Corp, Mountain View, Calif) containing isoproterenol (Iso) (60 mg/kg per day), angiotensin II (2 mg/kg per day), or PBS were surgically inserted in 2-month-old mice as described previously. Swimming for 20 days as a model of exercise-induced cardiac hypertrophy was described previously.

### Statistical Analysis

All results are presented as means±SEM. Statistical analysis was performed using SigmaPlot 11.0 software for unpaired t test (for 2 groups) and 2-way ANOVA (for 4 to 6 groups).

### Results

#### Generation of IP$_3$ Receptor Transgenic Mice

To determine the role of IP$_3$-mediated Ca$^{2+}$ release in the heart, we generated inducible, cardiac-specific transgenic mice expressing the mouse type-2 IP$_3$R. Responder transgenic lines were crossed with driver transgenic mice encoding the tetracycline transactivator (tTA) protein (double transgenic [DTG]) and a “high” (3.11) and “low” (4.9) line were selected based on Western blot analysis (Figure 1A). TAC induced a 2.4-fold increase in IP$_3$R2 protein expression in the mouse heart, and relative to this, lines 3.11 and 4.9 showed 12- and 5-fold more protein expression, respectively. To examine the reliability of the inducible system, doxycy-
cine (Dox) was administered to the low-expressing DTG mice, causing near complete extinguishment of IP$_3$R$_2$ expression (Figure 1B). Importantly, ventricular myocytes isolated from adult IP$_3$R$_2$ DTG mice showed a significant increase of amplitude in the Ca$^{2+}$ transient and extrasystolic Ca$^{2+}$ transients after ET-1 stimulation, whereas no effect was observed in tTA control ventricular myocytes (Figure 1C). Immunocytochemistry from DTG ventricular myocytes showed that the IP$_3$R$_2$ was localized in a distinct sarcomeric pattern that is consistent with the SR (Figure 1D). Because of low endogenous expression, little IP$_3$R$_2$ protein was detectable in control tTA ventricular myocytes (Figure 1D). The IP$_3$R$_2$ antibody used also reacted with a nucleoplasmic protein in all samples, masking the nuclear envelope localized IP$_3$R$_2$.

High-Expressing IP$_3$R$_2$ Transgenic Mice Display Cardiac Hypertrophy

Both high and low IP$_3$R$_2$ DTG mice (no Dox) developed mild cardiac hypertrophy by 3 months of age (Figure 2A). Consistent with this phenotype, reexpression of the hypertrophy-related genes atrial natriuretic factor and skeletal α-actin were observed in the hearts of high-expressing DTG mice (Figure 2B). DTG mice demonstrated normal values of baseline ventricular performance by echocardiography (Figure 2C), baseline myocyte Ca$^{2+}$ transient amplitudes, and SR Ca$^{2+}$ content in isolated cells (data not shown). However, a more sensitive approach using a Millar catheter showed that high-expressing IP$_3$R$_2$ DTG mice had a reduced contractile response to dobutamine infusion, although no change in baseline function was observed (Figure 2D). Myocyte Ca$^{2+}$ responses following Iso stimulation were also reduced, suggesting desensitization of β-adrenergic receptors (data not shown). Thus, outside of a mild deficit in functional reserve, high-expressing DTG mice showed no greater signs of heart disease past 10 months of age.

To further investigate the hypothesis that the IP$_3$R$_2$ was involved in hypertrophic signaling, high-expressing DTG and littermate tTA mice were subjected to 2 weeks of TAC to induce hypertrophy. Indeed, high-expressing DTG mice at 9 to 10 weeks of age displayed enhanced cardiac hypertrophy after TAC with equivalent pressure gradients across the constrictions (Figure 2E and data not shown). Assessment of myocyte cross sectional areas also showed significantly more hypertrophy in IP$_3$R$_2$ DTG mice compared to tTA controls (Figure 2F).

As previously reported, β-adrenergic stimulation activates protein kinase (PK)A, leading to IP$_3$R$_2$ phosphorylation and sensitized Ca$^{2+}$ release to IP$_3$. Consistent with these observations, 2 weeks of Iso infusion with Alzet minipumps produced more cardiac hypertrophy in DTG mice compared with tTA controls (Figure 2G and 2H). To further examine the impact of increased IP$_3$R$_2$ expression downstream of GPCR signaling, we crossed IP$_3$R$_2$ DTG mice with transgenic mice overexpressing Gaq, a direct PLC effector and disease inducer. As predicted, combined IP$_3$R$_2$ and Gaq overexpression exacerbated the hypertrophic phenotype and pathology, such as greater increases in ventricle weight normalized to body weight and decreased fractional shortening assessed by echocardiography (Figure 2I and 2J). The inset in Figure 2I shows that Gaq protein was overexpressed by ~3-fold with the transgene when crossed into the IP$_3$R$_2$ DTG background. Taken together, these results suggest that IP$_3$R$_2$ expression can enhance cardiac hypertrophy and disease downstream of multiple pathological stimuli.

Low-Expressing IP$_3$R$_2$ Transgenic Mice Show Increased Cardiac Hypertrophy to Iso and Exercise

We also repeated our entire analysis with low-expressing IP$_3$R$_2$ DTG mice. Remarkably, 2 weeks of TAC stimulation in low-expressing IP$_3$R$_2$ DTG mice failed to show augmented cardiac hypertrophy compared to tTA control mice (Figure 3A and 3B). Pressure gradients across the aortic constriction...
were not different between the 2 groups of TAC mice (data not shown). However, low-expressing IP3R2 DTG mice did show significantly greater cardiac hypertrophy following 2 weeks of Iso infusion, similar to the enhancement observed in high-expressing DTG mice (Figure 3C and 3D). In addition, the enhanced hypertrophic response in low-expressing DTG mice caused by \(\beta\)-adrenergic stimulation was reversed when the transgene was shut down by administration of Dox.

Figure 2. High-expressing IP3R2 DTG mice show greater cardiac hypertrophy. A, Heart weight (HW) normalized to body weight (BW) for control (tTA) and IP3R2 DTG (both lines) mice at 3 months of age. \(*P<0.05\) vs tTA controls. B, RT-PCR for atrial natriuretic factor (ANF), skeletal \(\alpha\)-actin (\(\alpha\)SkA) or L7 (control) from hearts of control (tTA) and IP3R2 DTG (both lines) mice at 3 months of age. Twenty-five and 28 cycles of PCR are shown. Two mice were used for each of the 3 groups. C, Fractional shortening (FS) by echocardiography in control (tTA) and IP3R2 DTG (line 3.11) mice at 3 months of age. D, Cardiac contractility (+dP/dt) with or without dobutamine (32 ng/kg per minute) assessed by invasive hemodynamics in control (tTA) and IP3R2 DTG (line 3.11) mice at 4 months of age. \(\dagger P<0.05\) vs no dobutamine; \(\ddagger P<0.05\) vs tTA with dobutamine. E, HW/BW in control (tTA) and IP3R2 DTG (line 3.11) mice 2 weeks after sham or TAC. \(\ddagger P<0.05\) vs sham; \(\ddagger P<0.05\) vs tTA TAC. F, Histological analysis of myocyte cross-sectional areas from ventricles of the indicated groups of mice 2 weeks after sham or TAC. G, HW/BW in the indicated groups of mice 2 weeks after Iso infusion or saline (Veh). \(\dagger P<0.05\) vs vehicle; \(\ddagger P<0.05\) vs tTA Iso. H, Histological analysis of myocyte cross-sectional areas from ventricles of the indicated groups of mice 2 weeks after Iso/saline infusion. I, Ventricular weight (VW) normalized to BW for control (tTA) and IP3R2 DTG (line 3.11) mice crossed with Gq transgenic mice at 3 months of age. Inset, Western blot for Gq protein in the hearts of the indicated mice. \#P<0.05 compared with other genotypes. J, FS in the indicated genotypes of mice at 8 weeks of age measured by echocardiography. \#P<0.05 compared with other genotypes. Numbers in the bars indicate mice used or sample sizes. All mice were fully induced for transgene expression (no Dox).

Figure 3. Low-expressing IP3R2 DTG mice demonstrate enhanced cardiac hypertrophy only to Iso infusion and swimming. A and B, Heart weight/body weight ratio (HW/BW) and myocyte cross-sectional areas from control (tTA) and IP3R2 DTG (line 4.9) mice 2 weeks after sham or TAC without Dox (induced). \(\dagger P<0.05\) vs sham. C and D, HW/BW and myocyte cross-sectional areas in the indicated groups (DTG, line 4.9) of mice 2 weeks after Iso infusion or vehicle (Veh), without Dox (induced). \(\dagger P<0.05\) vs vehicle; \(\ddagger P<0.05\) vs tTA Iso. E and F, HW/BW and myocyte cross-sectional areas for control (tTA) and IP3R2 DTG (line 4.9) mice 2 weeks after Iso/vehicle infusion with Dox present in the drinking water (transgene off). G and H, HW/BW after 3 weeks of swimming exercise in tTA or IP3R2 DTG (line 4.9 and line 3.11) without Dox (induced). \(\dagger P<0.05\) vs rest; \(\ddagger P<0.05\) vs tTA swim. Numbers in the bars indicate mice or samples analyzed.
Inhibition of IP3 Signaling Reduces Cardiac Hypertrophy to Iso and Angiotensin II Infusion

Here, we generated inducible IP3-sponge transgenic mice to examine its necessity in programming hypertrophy. The IP3-sponge is a truncated and soluble IP3 receptor that binds free IP3 with exceedingly high affinity. Two responder transgenic lines were obtained, although only the higher expressing line (line 22.3) was analyzed (Figure 4A). To determine the effectiveness of IP3-chelating activity in vivo, we crossed IP3-sponge mice with high-expressing IP3R2 DTG mice, after which adult myocytes were isolated and stimulated with ET-1. Importantly, triple transgenic mice (tTA driver, IP3-sponge, and IP3R2 responder transgenes) showed no loss in protein expression for either the IP3-sponge or IP3R2 (Figure 4B). Myocytes from IP3R2 high-overexpressing mice showed a robust increase in Ca2+ transients and arrhythmic events, which was completely blocked by the IP3-sponge (Figure 4C and 4D). Thus, targeted expression of the IP3-sponge in the mouse heart eliminates functional IP3-dependent Ca2+ release in isolated ventricular myocytes.

To assess the impact of IP3 signaling inhibition on pressure-overload hypertrophy, IP3-sponge DTG and tTA control mice were subjected to TAC stimulation. Consistent with the results observed in low-expressing IP3R2 DTG mice, IP3-sponge overexpression showed no ability to reduce the cardiac hypertrophic response after 2 weeks of TAC stimulation compared with tTA controls (Figure 4E and 4F). However, IP3-sponge DTG mice showed significantly less hypertrophy following 2 weeks of Iso infusion (Figure 4G and 4H). No reduction in fractional shortening was noted over 2 weeks of Iso infusion, and levels of induced fibrosis were similar between tTA and IP3-sponge DTG hearts (Online Figure II). We also observed that the hypertrophic response caused by angiotensin II infusion was significantly reduced in IP3-sponge DTG mice (Figure 4I). Taken together, these results indicate that IP3 signaling plays a necessary role in selective forms of hypertrophic stimulation in adult mouse heart (see Discussion).

Calcineurin Signaling Underlies IP3-Regulated Hypertrophy

Calcineurin is Ca2+/calmodulin-dependent protein phosphatase that functions as a central regulator of cardiac hypertrophy, in part, by activating a transcription factor family known...
as NFAT (nuclear factor of activated T cells).31 Here, we hypothesized that the calcineurin/NFAT pathway might respond to IP3R signaling in mediating the cardiac hypertrophic response. To address this issue, high and low IP3R2 DTG mice were crossed with NFAT-luciferase reporter transgenic mice, showing a 3.5- and 2.4-fold increase in NFAT activity, respectively, compared with tTA mice at 8 weeks of age (Figure 5A). Expression of the IP3-sponge did not affect NFAT-luciferase activity at baseline in the heart (Figure 5A).

To extend these results and determine whether calcineurin was necessary for IP3R2 enhanced hypertrophy, we crossed the IP3R2 responder and tTA driver transgenes into the CnAβ-null background. CnAβ+/− (wild-type) controls were also generated from the same backcross. Importantly, cardiac expression level of IP3R2 protein directed by the transgene also generated from the same backcross. Importantly, cardiac expression level of IP3R2 protein directed by the transgene was similar between the CnAβ−/− and CnAβ+/− backgrounds (Figure 5B). Remarkably, the augmented hypertrophy profile observed in IP3R2 DTG mice following 2 weeks of Iso stimulation was blocked in the CnAβ−/− background but, once again, was significantly augmented in the wild-type background (Figure 5C and 5D). Collectively, these results indicate that calcineurin/NFAT serve as a downstream effector of IP3R-mediated Ca2+ signaling in the heart.

Discussion

IP3Rs are intracellular ligand-gated Ca2+ release channels that are activated by IP3 binding, where they function downstream of growth factors and GPCR signaling events.6 The physiological role of Ca2+ release from the IP3R in ventricular myocytes has been a controversial issue, with some reports suggesting no effect on ECC, whereas others have observed a small but significant effect on spontaneous Ca2+ release in the form of sparks and enhanced Ca2+ transients.15,52–34 In atrial myocytes, IP3-mediated Ca2+ release appears to be a more prominent event that modulates ECC and SR Ca2+ release,55,56 likely because of higher endogenous IP3R expression levels.15,54 Consistent with these reports, transgene-directed overexpression of IP3R2 in ventricular myocytes had a prominent effect on ECC and even arrhythmia on ET-1 stimulation, further suggesting that IP3Rs are positioned within the junctional SR, where they can affect ryanodine receptor (RyR) activity.

To affect ECC, the IP3Rs need to be positioned within the proper functional domains of the SR. Previous analysis of this issue demonstrated that IP3R2 is enriched at the nuclear envelope, which is contiguous with the SR/ER network in adult cardiac myocytes.12,37,38 However, localization to the nuclear envelope should not affect ECC but, instead, appears to control nuclear Ca2+ signaling39 and a local pool of CaMKII.5 In addition to the nuclear envelope, the IP3Rs are prominently localized to the SR, in similar regions as the RyR.16 Indeed, immunohistochemistry of adult myocytes from IP3R2 DTG overexpressing mice versus tTA controls unequivocally showed IP3R2 localization within the entire expanse of the SR and around the nucleus.

Another aspect of the controversy surrounding a functional role for IP3Rs in cardiac myocytes is that generation of IP3 appears to be relatively weak compared with other cell types.40,41 For example, cardiomyocytes from mouse or human display only a 1.5- to 2-fold activation of PLC in response to α1-adrrenergic receptor stimulation, achieving a level of ~30 nmol/L.42–45 However, phosphorylation of IP3R2 by PKA sensitizes the channel and enhances IP3-mediated Ca2+ release at lower IP3 concentrations.30 In addition to this mechanism, Iso stimulation in cultured cardiomyocytes enhances IP3 generation through an ET-1 paracrine/autocrine signaling circuit.38 β-Adrenergic stimulation also enhances SR Ca2+ levels and sensitizes the RyR, together leading to augmented Ca2+ release. In support of this contention, low levels of ET-1, which did not change Ca2+ release or induce arrhythmia, did synergistically increase arrhythmia when forskolin was also used to elevate cAMP. We also believe that physiological exercise-induced cardiac hypertrophy, which appears to elicit a strong fear response in mice, augmented IP3R Ca2+ release through an associated β-adrenergic costimulation effect. These concepts are also consistent with the vast array of neurohumoral mediators that underlie pathological cardiac hypertrophy and failure, where multiple Gαq-coupled receptor agonists likely synergize with cAMP elevation afforded by β-receptor signaling to induce pathology.

Interestingly, only the high IP3R2 DTG line showed augmented cardiac hypertrophy with pressure-overload stimulation. In contrast, the low overexpressors and the IP3-sponge mice each failed to show an effect with pressure-overload stimulation. The simplest interpretation of these results is that a requirement for endogenous IP3Rs is easily bypassed with extreme hypertrophic stimulation, such as afforded by pressure overload. Indeed, TRPC channels are also activated in pressure-overloaded hearts, where they could easily compensate and provide local Ca2+ entry in the...
absence of IP₃R signaling to maintain calcineurin activation. In contrast, Iso infusion engages a more restricted set of signaling pathways, such as PKA activation through elevated cAMP. As discussed earlier, β-adrenergic/PKA stimulation uniquely primes IP₃R activation and Ca²⁺ release, possibly explaining why Iso infusion in low-expressing DTG mice and in IP₃R-sponge mice demonstrated a positive effect versus tTA control mice.

The increase in Ca²⁺ release mediated by IP₃Rs at the level of the SR could induce the hypertrophic response through a number of different signaling mechanisms. Our working hypothesis is that IP₃R Ca²⁺ release generates a local Ca²⁺ signaling effect at the level of the T-tubular–SR junctional complex. Indeed, calcineurin is anchored at the Z-lines to calsscin and α-actinin, in immediate proximity to the SR junctions. Calcineurin was also shown to respond to IP₃-mediated Ca²⁺ release from a perinuclear/nuclear location in neonatal cardiomyocytes to induce hypertrophy. Consistent with these results, we observed prominent NFAT activation in IP₃R2 DTG hearts and that deletion of CNaB blocked the ability of Iso to enhance hypertrophy through the IP₃R2 transgene. This IP₃R-dependent release of Ca²⁺ at the T-tubular–SR junctions could also stimulate RyR Ca²⁺ leak, providing additional regional Ca²⁺ elevations to affect calcineurin. Thus, antagonism of the IP₃R may be a clinically relevant target, because it might reduce both arrhythmia and hypertrophic growth propensity.

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Disclosures

None.

References

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**Novelty and Significance**

**What Is Known**
- IP₃R signaling has been implicated in regulating cardiac hypertrophy.
- IP₃R-mediated calcium release has been implicated in regulating excitation–contraction coupling in the heart.
- IP₃ signaling has been implicated in regulating calcineurin/NFAT signaling.

**What New Information Does This Article Contribute**
- We provide the first description of IP₃R2 overexpression in the heart of transgenic mice, which definitively shows that it can regulate cardiac hypertrophy.
- We provide the first description of IP₃ inhibition in the mouse heart and the observation that this mediator is required for isoproterenol induced hypertrophy.
- We provide the first proof that calcineurin/NFAT signaling mediates IP₃R2 signaling in vivo to control cardiac hypertrophy.

This study was designed to evaluate the importance of IP₃R2 calcium release and signaling in mediating the cardiac hypertrophic response in vivo. We examined both pressure overload–induced hypertrophy and neurohormonal-regulated hypertrophy in IP₃R2 TG mice and mice expressing the IP₃-sponge to block this calcium release pathway. Our results definitively show that IP₃R2-mediated calcium release in response to neurohormonal stimulation can underlie the cardiac hypertrophic response, in part, by activating calcineurin/NFAT signaling.
The IP₃ Receptor Regulates Cardiac Hypertrophy in Response to Select Stimuli
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Online Figure I. Assessment of elevated cAMP on Ca^{2+} handling in IP_{3}R2 DTG hearts with or without a small ET-1 stimulus at 40 nM. (A,B) Percent increase in the amplitude of the Ca^{2+} transient after forskolin (A) or ET-1 co-stimulation (B) in isolated adult cardiomyocytes from control tTA or IP_{3}R2 DTG hearts. Forskolin equally increased the Ca^{2+} transient in both genotypes, although co-stimulation with ET-1 gave a larger effect in the DTG myocytes. (C) Arrhythmic Ca^{2+} transients (%) in adult myocytes from DTG hearts with forskolin or forskolin with ET-1. ET-1 was used at 40 nM which is too low to induce arrhythmia in wildtype or IP_{3}R2 myocytes, but it can with forskolin in IP_{3}R2 DTG myocytes. (D,E) Representative Fura-2 ratio fluorescence tracings from IP_{3}R2 DTG (line 3.11) adult cardiomyocytes with forskolin (D) or forskolin with low ET-1 (E). Arrow shows compound addition.
Online Figure II. IP₃-sponge expression and suppression of IP₃R signaling does not promote pathologic hypertrophy with isoproterenol infusion. (A) Echocardiography to assess ventricular fractional shortening (FS) in tTA controls and IP₃-sponge DTG mice infused with vehicle or Iso for 2 weeks. No reductions in FS were observed in any group. Number of mice analyzed is shown in the bars. (B) Metamorph quantitation of ventricular fibrosis from Masson’s trichrome-stained histological sections in the mice treated as described in A. *P<0.05 versus tTA Veh. The IP₃-sponge did not worsen fibrosis with ISO infusion.
SUPPLEMENTAL MATERIALS AND METHODS

Generation of transgenic mice. cDNAs encoding mouse IP3R2 and recombinant Flag-tagged IP3-sponge protein 1,2 were cloned into the murine inducible α-myosin heavy chain (α-MHC) promoter expression vector 3 (gift from Dr. Jeffrey Robbins, Children’s Hospital, Cincinnati, Ohio, USA). Gcαq transgenic mice were kindly provided by Dr. Gerald Dorn II 4. NFAT-luciferase reporter transgenic mice and calcineurin Aβ null mice (CnAβ−) were previously reported 5,6. To reduce strain effects the CnAβ null mice (C57BL/6) were backcrossed 6 generations into the IP3R2 (FVB/N) background. Mice were given doxycycline (Sigma, Saint Louis, MO) at 1g/L in the drinking water for 3 weeks to shutdown protein expression from the inducible transgenes. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center.

Western blot analysis. Western blot analysis for mouse ventricle homogenates were performed as previously reported 7. Antibodies included IP3R2, Gcαq and α-tubulin (Santa Cruz), GAPDH (Research Diagnostics Inc., Flanders, NJ) and Flag M2 monoclonal (Sigma). Chemifluorescent detection was performed with the Vistra ECF reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and scanned with a Storm 860 PhosphorImager (Molecular Dynamics, Piscataway, NJ).

Isolation of adult cardiomyocytes and Ca2+ measurements. Only Ca2+-tolerant cardiomyocytes were selected for Ca2+ measurements as previously reported 8. Ca2+ transients were measured using Fluo-4 as previously described 9 or using Fura-2 fluorescence ratio at room temperature using a Delta Scan dual-bean spectrofluorophotometer (Photon Technology, Birmingham, NJ, USA), at an emission wavelength of 510 nM and excitation of 340 and 380 nM. The amplitude (Δ340/380 nM ratio) of the Ca2+ transients was measured before and after exposure to freshly prepared 100 nM endothelin-1 (ET-1, Calbiochem) or with 1 µM forskolin alone or in combination with 40 nM ET-1. Arrhythmias were classified by three or more extra-systolic Ca2+ release events over a 15 min recording period following previously reported criteria 10.

RT-PCR, luciferase assay, immunohistochemistry, and histological analysis. Reverse-transcriptase PCR was performed as previously described 11. For histological analysis, hearts were collected at the indicated times, fixed in 10% formalin containing PBS, and embedded in paraffin. Myocyte cross-sectional areas were analyzed in slides stained with wheat-germ agglutinin-FITC 8. Immunohistochemistry on adult myocytes was performed as described previously using an IP3R2 antibody (Abcam) and α-actinin (Sigma) 12.

Echocardiography, invasive hemodynamics, and surgical models. Mice were anesthetized with isoflurane, and echocardiography was performed using a Hewlett Packard 5500 instrument with a 15-MHz microprobe as previously described 7. Invasive hemodynamics in the closed-chest mouse with a 1.4F Millar catheter was performed as described previously 13. Transverse aortic constriction (TAC) to induce cardiac pressure overload hypertrophy was previously described 7. Pressure gradients across the constriction were measured by Doppler echocardiography as previously described 14. Alzet miniosmotic pumps (no. 2002; Alza Corp., Mountain View, California, USA) containing isoproterenol (Iso) (60 mg/kg/day), angiotensin II (2 mg/kg/day) or PBS were surgically inserted in 2 month-old mice as previously described 11. Swimming for 20 days as a model of exercise-induced cardiac hypertrophy was described previously 5,8.

Statistical analysis. All results are presented as means plus or minus SEM. Statistical analysis was performed using SigmaPlot11.0 software for unpaired t-test (for two groups) and two-way ANOVA (for four-six groups).
References