Dysfunctional ryanodine receptor and cardiac hypertrophy: role of signaling molecules

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1Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina; and 2Division of Molecular Cardiovascular Biology, Cincinnati Children’s Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio

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Yamaguchi N, Chakraborty A, Pasek DA, Molkentin JD, Meissner G. Dysfunctional ryanodine receptor and cardiac hypertrophy: role of signaling molecules. Am J Physiol Heart Circ Physiol 300: H2187–H2195, 2011. First published March 18, 2011; doi:10.1152/ajpheart.00719.2010.—Mice with three amino acid mutations in the calmodulin binding domain of type-2 ryanodine receptor ion channel (Ryr2<sup>ΔADA/ΔADA</sup> mice) have impaired intracellular Ca<sup>2+</sup> handling and cardiac hypertrophy with death at an early age. In this report, the role of signaling molecules implicated in cardiac hypertrophy of Ryr2<sup>ΔADA/ΔADA</sup> mice was investigated. Calcineurin A-β (CNA-β) and nuclear factor of activated T cell (NFAT) signaling were monitored in mice carrying either luciferase transgene driven by NFAT-dependent promoter or knockout of CNA-β. NFAT transcriptional activity in Ryr2<sup>ΔADA/ΔADA</sup> hearts was not markedly upregulated at embryonic day 16.5 compared with wild-type but significantly increased at postnatal days 1 and 10. Ablation of CNA-β extended the life span of Ryr2<sup>ΔADA/ΔADA</sup> mice and enhanced cardiac function without improving sarcoplasmic reticulum Ca<sup>2+</sup> handling or suppressing the expression of genes implicated in cardiac hypertrophy. Embryonic day 16.5 Ryr2<sup>ΔADA/ΔADA</sup> mice had normal heart weights with no major changes in Akt1 and class II histone deacetylase phosphorylation and myocyte enhancer factor-2 activity. In contrast, phosphorylation levels of Erk1/2, p90 ribosomal S6 kinases (p90RSKs), and GSK-3β were increased in hearts of embryonic day 16.5 homozygous mutant mice. The results indicate that an impaired calmodulin regulation of RyR2 was neither associated with an altered CNA-β/NFAT, class II histone deacetylase (HDAC)/MEF2, nor Akt signaling in embryonic day 16.5 hearts; rather increased Erk1/2 and p90RSK phosphorylation levels likely leading to reduced GSK-3β activity were found to precede development of cardiac hypertrophy in mice expressing dysfunctional ryanodine receptor ion channel.

Cardiac hypertrophy and heart failure are complex diseases that develop in response to physiological and pathological stimuli (13, 16, 20, 21, 28, 46). Physiological hypertrophy induced by exercise training is not associated with overt cardiac dysfunction and can be reversed by normal cardiac workloads. Physiological cardiac hypertrophy is thought to be largely controlled through activation of receptor tyrosine kinases that regulate protein synthesis via Akt/PKB signaling. In contrast, pathological hypertrophy occurs in response to prolonged mechanical stress or abnormal neurohormonal activation and has been studied in genetically modified mice subjected to transaortic banding and hormone stimuli. Pathological cardiac growth is triggered via signaling pathways that in many cases are activated by temporal imbalances in cellular Ca<sup>2+</sup> concentration.

Pathological cardiac hypertrophy may be triggered via the entry of Ca<sup>2+</sup> through canonical transient receptor potential channels (TRPCs) (8, 48) and by hormones that cause Ca<sup>2+</sup> release from inositol-1,4,5-trisphosphate (IP<sub>3</sub>)-sensitive stores (13). Ca<sup>2+</sup> plays a critical role in cardiac hypertrophy by binding calmodulin (CaM) and protein kinases C. One proposed pathway involves calcineurin (CN), a Ca<sup>2+</sup>/CaM-activated serine/threonine phosphatase that dephosphorylates nuclear factor of activated T cell (NFAT), a transcription factor implicated in pathological cardiac hypertrophy (20, 21). A second signaling mechanism involves the activation of G<sub>q11</sub>-coupled receptors that lead to the activation of phospholipase C-β and generation of diacylglycerol, an activator of protein kinases C, and IP<sub>3</sub>, which binds to its nuclear receptor to cause Ca<sup>2+</sup> release in the nucleus and activation of nuclear CaMKIIβ. Phosphorylation of class II histone deacetylases (HDACs) by CaMKII and protein kinases C and D activates myocyte enhancer factor 2 (MEF2) that regulates cardiac gene expression (28).

Ryanodine receptors (RyR2) are large ion channels comprised of four 560-kDa RyR subunits and four 12.6-kDa FK506 binding proteins (FKBP12.6). Multiple endogenous effectors that regulate RyR2 include Ca<sup>2+</sup>, Mg<sup>2+</sup>, ATP, and associated proteins such as protein kinases, phosphatases, and CaM (14, 15, 29). RyR2s are important for normal cardiac function by releasing Ca<sup>2+</sup> from the sarcoplasmic reticulum (SR) required for muscle contraction. Aberrant function of RyR2 has been implicated in cardiac hypertrophy and heart failure. For example, PKA-mediated phosphorylation of RyR2 was reported to cause release of the small FKBP12.6 (calstabin 2), leading to a leaky SR Ca<sup>2+</sup> channel, aberrant contractile function, and heart failure in animal models and patients (46). Abnormal SR Ca<sup>2+</sup> handling and associated cardiac myopathies were also observed in patients and mice carrying RyR2 mutations associated with catecholaminergic polymorphic ventricular tachycardia (17). We identified three amino acid substitutions in the CaM binding domain of RyR2 (RyR2<sup>F3603A</sup> or RyR2<sup>W3587A/L3591D</sup>) that eliminate inhibition of RyR2 activity by CaM at both diastolic and systolic Ca<sup>2+</sup> concentrations (49). Homozygous mice expressing the CaM-defective mutant form of RyR2 (RyR2<sup>ΔADA/ΔADA</sup> mice) had increased heart-to-body weight ratios at postnatal day 1 and died within 16 days of birth. Sustained Ca<sup>2+</sup> transients revealed abnormal Ca<sup>2+</sup> handling in cultured homozygous mutant cardiomyocytes. Biochemical studies indicated upregulation of genes or enzyme activities associated with class II histone deacetylase (class II...
NFAT participate in but are not essential for cardiac hypertrophy in mice expressing a dysfunctional ryanodine receptor.

**MATERIALS AND METHODS**

**Materials.** [H]ryanodine was obtained from Perkin Elmer Life Sciences, and protease and phosphatase inhibitor cocktails were from Sigma. HDAC5 rabbit polyclonal antibody was from Biovision. pHDAC4-Ser246/HDAC5-Ser259/HDAC9-Ser220 rabbit polyclonal antibody was from GenScript. GSK-3β rabbit monoclonal antibody was from GenScript. Akt, HDAC5 rabbit polyclonal antibody was from Biovision. FVB. All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

**Preparation of heart homogenates.** Echocardiography was performed using mice with a hybrid genetic background of 129svev and FVB. All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

** Luciferase reporter assay.** Whole hearts (ventricles and atria) of 1- and 10-day-old mice and ventricles (hearts without atria) of E16.5 mice were homogenized using a Tekmar Tissumizer for 3 × 7 s at a setting of 13,500 rpm or glass homogenizer, respectively, in 20 mM imidazole (pH 7.0), 0.3 M sucrose, 0.1 M NaCl, protease and phosphatase inhibitor cocktails, 25 mM β-glycerophosphate, 5 mM NaF, and 2.5 mM NaVO₄. Homogenates were stored in small aliquots at −80°C. Protein concentrations were determined using bichinchoninic acid assay.

**Western blot analyses.** Homogenates (20 μg protein/lane) were separated by 10% SDS/PAGE, transferred to nitrocellulose membranes, and probed with rabbit monoclonal or polyclonal antibodies. Western blots were developed using enhanced chemiluminescence reagents. Membranes were probed with rabbit monoclonal or polyclonal antibodies. Proteins were detected using chemiluminescence reagents.

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Although embryonic expression changes as early as E16.5. mRNA expression levels of TRPC1 increased 1.35-fold in hearts of E16.5 Ryr2ADA/ADA mice, which suggests that cardiac gene remodeling begins before postnatal day 1.

Ryr2ADA is associated with upregulation of genes in embryonic day 16.5 hearts. To confirm early changes in cardiac gene expression, quantitative RT-PCR was performed on embryonic day 16.5 (E16.5) Ryr2ADA/ADA hearts. As shown in Fig. 1, ANP mRNA levels increased 2.5-fold in hearts of E16.5 Ryr2ADA/ADA mice compared with WT, whereas expression of two other cardiac hypertrophy associated genes, β-MHC and BNP, was not significantly increased.

Canonical TRPC channels TRPC1, TRPC3, and TRPC6 promote cardiomyocyte hypertrophy and heart failure through the activation of calcineurin/NFAT signaling (8, 24, 31, 33, 40, 48). mRNA expression levels of TRPC1 increased 1.25-fold in Ryr2ADA/ADA hearts, whereas TRPC3 and TRPC6 mRNA levels were unchanged (Fig. 1). At E16.5, heart protein content was unchanged [0.272 ± 0.012 mg for Ryr2ADA/ADA (n = 16) vs. 0.272 ± 0.010 mg for WT (n = 16)]. The results suggest that although embryonic Ryr2ADA/ADA heart protein content, and thus heart weight, was unchanged, there was evidence for gene expression changes as early as E16.5.

Class II HDAC/MEF2 signaling in E16.5 hearts. To determine whether class II HDAC/MEF2 signaling was upregulated in E16.5 Ryr2ADA/ADA hearts, in vitro phosphorylation of a recombinant GST-HDAC4 fusion protein with CaMKII and PKC/D phosphorylation sites Ser467 and Ser632 was measured (4). No difference was observed in the phosphorylation of HDAC4 in lysates from homozygous and WT hearts (Fig. 2A). In immunoblot, no significant differences were observed in protein expression level of HDAC5 and phosphorylation level of HDAC4-Ser246/HDAC5-Ser259/HDAC9-Ser220 (Fig. 2B). Moreover, MEF2 transcriptional activity in E16.5 Ryr2ADA/ADA heart lysates was not increased (Fig. 2C). The results suggest that class II HDAC/MEF2 signaling was not upregulated in E16.5 Ryr2ADA/ADA hearts.

NFAT-dependent luciferase activity of E16.5 and postnatal Ryr2ADA/ADA mice. As part of a compensatory response, regulator of calcineurin 1 (Rcn1) is upregulated in hearts expressing a constitutively active form of calcineurin (50). We observed previously an increase in Rcn1 mRNA levels in 7- and 10-day-old but not in 1-day-old mutant hearts, which suggested a progressive increase in calcineurin signaling in postnatal Ryr2ADA/ADA hearts (49). To determine more directly the temporal changes of calcineurin/NFAT signaling, transgenic mice carrying the luciferase gene driven by NFAT-dependent promoter (47) were crossed with Ryr2+/ADA mice to obtain three genotypes (Ryr2+/-, Ryr2+/ADA, and Ryr2ADA/ADA) carrying the luciferase transgene. On days 1 and 10 of age, luciferase activities in homozygous Ryr2ADA/ADA hearts were 2 and 7.5 times higher than in WT hearts, respectively, with a modest but not significant change (1.4 times) at E16.5 (Fig. 3).

Knockout of CNA-β from Ryr2ADA/ADA mice. Calcineurin is composed of the 60-kDa catalytic subunit calcineurin A (CNA) and the 19-kDa regulatory subunit calcineurin B (CNB). Two isoforms of CNA, CNA-α and CNA-β, are expressed in cardiac muscle (37). Since previous work showed that calcineurin-β has a specific role in regulating hypertrophic growth in hearts (7, 42), Ryr2+/ADA mice were intercrossed with CNA-β knockout mice. Ryr2ADA/ADA/CNA-β+/- mice died between 6 and 17 days after birth (Fig. 4A) and exhibited an approximately twofold increase in heart weight (Fig. 4B) and heart-to-body weight ratio (Fig. 4C) at day 10 compared with WT mice, as reported previously (49). Homozygous ablation of CNA-β from Ryr2ADA/ADA mice prolonged the life span to between 14 to 50 days (Fig. 4A) and reduced the heart weight (Fig. 4B) and heart-to-body weight ratio (Fig. 4C) by ~25% and ~13%, respectively. Heterozygous ablation of CNA-β in postnatal Ryr2ADA/ADA hearts was associated with a ~10% increase in heart weight (Fig. 4B) and heart-to-body weight ratio (Fig. 4C) compared with WT mice, as reported previously (49). Since previous work showed that CNA-β has a specific role in regulating hypertrophic growth in hearts (7, 42), Ryr2+/ADA mice were intercrossed with CNA-β knockout mice. Ryr2ADA/ADA/CNA-β+/- mice died between 6 and 17 days after birth (Fig. 4A) and exhibited an approximately twofold increase in heart weight (Fig. 4B) and heart-to-body weight ratio (Fig. 4C) at day 10 compared with WT mice, as reported previously (49).

Table 1. Echocardiography of 1-day-old WT and mutant mice

<table>
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<tr>
<th>Parameters</th>
<th>RyR2+/+</th>
<th>RyR2+/ADA</th>
<th>RyR2ADA/ADA</th>
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<tr>
<td>HR, beats/min</td>
<td>490 ± 13</td>
<td>445 ± 31</td>
<td>353 ± 12*</td>
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<td>LVEDD, mm</td>
<td>1.29 ± 0.07</td>
<td>1.54 ± 0.09</td>
<td>1.61 ± 0.15</td>
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<td>LVESD, mm</td>
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<td>0.76 ± 0.08</td>
<td>1.09 ± 0.13</td>
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<td>FS, %</td>
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<td>51.2 ± 3.5</td>
<td>33.6 ± 2.3*</td>
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<td>LVPWD, mm</td>
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<td>0.50 ± 0.05</td>
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<td>LVFPWS, mm</td>
<td>0.75 ± 0.08</td>
<td>0.76 ± 0.07</td>
<td>0.67 ± 0.04</td>
</tr>
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</table>

Values are means ± SE; n = 3, 5, and 9 mice for RyR2+/+, RyR2+/ADA, and RyR2ADA/ADA groups, respectively. WT, wild-type; HR, heart rate; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening (LVEDD – LVESD)/LVEDD; IVSD, interventricular septum diastolic thickness; IVSS, interventricular septum systolic thickness; LVPWD, left ventricular posterior wall diastolic thickness; LVFPWS, left ventricular posterior wall systolic thickness. *P < 0.05 compared with RyR2+/+.

Fig. 1. Quantitative RT-PCR of cardiac hypertrophy associated genes in embryonic day 16.5 (E16.5) hearts of ryanodine receptors (Ryr)2+/+ , Ryr2+/ADA, and Ryr2ADA/ADA mice. mRNA levels were measured by quantitative RT-PCR and normalized to those of β-MHC, β-myosin heavy chain; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; TRPC, canonical transient receptor potential. Data are means ± SE of 10 samples. *P < 0.05 compared with Ryr2+/+.

Fig. 2. A) Expression of HDAC4 in lysates from homozygous and WT hearts. B) Western blot analysis of calcineurin/NFAT signaling in E16.5 Ryr2+/-, Ryr2+/ADA, and Ryr2ADA/ADA hearts. C) Western blot analysis of calcineurin/NFAT signaling in E16.5 Ryr2+/-, Ryr2+/ADA, and Ryr2ADA/ADA hearts.
Ryr2A/ADA mice resulted in a modest (not significant) increase of life span (Fig. 4A) and reduction in heart weight (64.3 ± 2.9 vs. 73.2 ± 5.5 mg for Ryr2A/ADA/CNA-β−/−; n = 19–24) and heart-to-body weight ratio (0.91 ± 0.03 vs. 1.00 ± 0.03 for Ryr2A/ADA/CNA-β−/−; n = 19–24).

The ventricle function of 10-day-old Ryr2A/ADA/CNA-β−/− (WT), Ryr2A/ADA/CNA-β−/− mice was compared by conscious M-mode echocardiography. The homozygous Ryr2A/ADA mutation decreased the heart rate of Ryr2A/ADA/CNA-β−/− (490 beats/min) and Ryr2A/ADA/CNA-β−/− (479 beats/min) mice compared to WT (589 beats/min) (Table 2). In Table 2, Ryr2A/ADA/CNA-β−/− mice had both left ventricular end-diastolic and end-systolic dimensions higher compared with WT, which resulted in significantly reduced fractional shortening (10.3% for Ryr2A/ADA/CNA-β−/−, 57% for WT mice). The result indicated dilated cardiomyopathy in Ryr2A/ADA/CNA-β−/− mice.

Elimination of CNA-β from WT mice (Ryr2A/ADA/CNA-β−/−, Table 2) caused a moderate decrease in left ventricular posterior wall systolic thickness, without significantly altering the other parameters. By comparison, knockout of CNA-β from Ryr2A/ADA/CNA-β−/− mice (Ryr2A/ADA/CNA-β−/−; Table 2) significantly reduced left ventricular end-diastolic and end-systolic dimensions. Fractional shortening caused by changes in left ventricular end-diastolic and end-systolic dimensions increased twofold (21.4% for Ryr2A/ADA/CNA-β−/− mice, 10.3% for Ryr2A/ADA/CNA-β−/− mice) but was still less than the 57% value for WT. The results indicate that ablation of CNA-β modestly improves cardiac function and thereby likely extends the life span of Ryr2A/ADA mice.

Bmax values of [3H]ryanodine binding, which are a measure of Ryr2 protein expression, and 45Ca2+ uptake rates, which are a measure of SR Ca2+ pump activity, were determined in 10-day-old heart homogenates of mice targeted for Ryr2A/ADA and CNA-β knockout. Ryr2A/ADA/CNA-β−/− heart homogenates had significantly reduced Bmax values of [3H]ryanodine binding (0.15 compared with 0.35 pmol/mg protein for WT) (Table 3), whereas 45Ca2+ uptake rates of Ryr2A/ADA/CNA-β−/− hearts were not significantly reduced (1.48 compared with 1.75 nmol/mg protein/min for WT). Ablation of CNA-β from Ryr2A/ADA mice further reduced Bmax values of [3H]ryanodine binding and 45Ca2+ uptake rates modestly. Similar small changes in [3H]ryanodine binding and Ca2+ uptake activities were also observed in heart homogenates of Ryr2A/ADA/CNA-β−/− mice compared with WT, which suggests that the effects of deleting CNA-β were not specific to the mutant mice. The results suggest that although ablation of CNA-β from Ryr2A/ADA mice improved cardiac function, it did not re-establish RyR expression or SR Ca2+ pump activity to WT levels.

The effects of deleting CNA-β from WT and mutant mice on mRNA levels of several cardiac hypertrophy associated genes were examined. In agreement with our previous finding (49), quantitative RT-PCR showed that mRNA levels for β-MHC, ANP, and BNP were upregulated in 10-day-old Ryr2A/ADA/CNA-β−/− mice compared with WT (Fig. 5). Knockout of
CNA-β in 10-day-old WT and RyR2<sup>ADA/ADA</sup> mice did not significantly alter expression of β-MHC, ANP, and BNP. mRNA levels of the second cardiac isoform CNA-α were modestly downregulated in RyR2<sup>ADA/ADA/CNA-β</sup> mice, whereas no differences were observed in the expression levels between WT and RyR2<sup>ADA/ADA/CNA-β</sup> mice. Thus genes implicated in cardiac hypertrophy were found to be upregulated in RyR2<sup>ADA/ADA</sup> mice even in the absence of CNA-β.

Other signaling pathways associated with cardiac hypertrophy. The phosphorylation status of GSK-3α and β and upstream protein kinases Akt1 and p90RSKs were examined in E16.5 RyR2<sup>ADA/ADA</sup> hearts. GSK3 is a serine/threonine kinase with two structurally similar isoforms, α and β. An unusual feature of these kinases is that phosphorylation reduces their activity. Studies with knock-in mice suggest that phosphorylation of GSK-3β-Ser9 leads to pathological cardiac hypertrophy during pressure overload, whereas phosphorylation of GSK-3α-Ser21 has a compensatory role (27). We observed a significant 1.5-fold increase in GSK-3β-Ser9 phosphorylation and a more modest (1.15-fold, significant) increase in GSK-3α-Ser21 phosphorylation in E16.5 RyR2<sup>ADA/ADA</sup> hearts compared with WT (Fig. 6, A and B). Total GSK-3β expression levels were unchanged. The results suggest that enhanced phosphorylation of GSK-3β may contribute to increased cardiac growth of postnatal mutant mice.

Table 3. Calcium handling by membrane fractions isolated from 10-day-old hearts of mice double targeted for RyR2<sup>ADA/ADA</sup> and CNA-β

<table>
<thead>
<tr>
<th>Genotype</th>
<th>B&lt;sub&gt;max&lt;/sub&gt; of [3H]ryanodine Binding, pmol/mg protein</th>
<th>&lt;sup&gt;45&lt;/sup&gt;Ca&lt;sup&gt;2+&lt;/sup&gt; Uptake Rate, nmol·mg protein&lt;sup&gt;−1·min&lt;/sup&gt;−1</th>
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<tr>
<td>RyR2&lt;sup&gt;++/++&lt;/sup&gt;</td>
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</tr>
<tr>
<td>CNA-β&lt;sup&gt;++/+&lt;/sup&gt; (WT)</td>
<td>0.35 ± 0.04</td>
<td>1.75 ± 0.19</td>
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<td>CNA-β&lt;sup&gt;+/-&lt;/sup&gt;</td>
<td>0.29 ± 0.05</td>
<td>1.64 ± 0.27</td>
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<tr>
<td>RyR2&lt;sup&gt;ADA/ADA&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>CNA-β&lt;sup&gt;++/+&lt;/sup&gt; (WT)</td>
<td>0.15 ± 0.02*</td>
<td>1.48 ± 0.22</td>
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<td>CNA-β&lt;sup&gt;+/-&lt;/sup&gt;</td>
<td>0.12 ± 0.01*</td>
<td>1.38 ± 0.18</td>
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Values are means ± SE in 5–7 experiments. *P < 0.05 compared with RyR2<sup>++/+</sup>/CNA-β<sup>++/+</sup>.

Table 2. Echocardiography of 10-day-old WT and mutant mice

<table>
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<tr>
<th>Parameters</th>
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<th>CNA-β&lt;sup&gt;++/+&lt;/sup&gt;</th>
<th>RyR2&lt;sup&gt;ADA/ADA&lt;/sup&gt;</th>
<th>CNA-β&lt;sup&gt;++/+&lt;/sup&gt;</th>
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<tr>
<td>HR, beats/min</td>
<td>589 ± 21</td>
<td>607 ± 18</td>
<td>490 ± 18*</td>
<td>479 ± 4*†</td>
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<td>LVEDD, mm</td>
<td>1.59 ± 0.11</td>
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<td>3.28 ± 0.24†</td>
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<td>LVESD, mm</td>
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<td>0.67 ± 0.06</td>
<td>2.96 ± 0.25†</td>
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<td>FS, %</td>
<td>57.0 ± 2.0</td>
<td>54.2 ± 2.8</td>
<td>10.3 ± 1.3*</td>
<td>21.4 ± 0.9*‡</td>
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<td>IVSD, mm</td>
<td>0.83 ± 0.07</td>
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<td>LVPWS, mm</td>
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<td>0.96 ± 0.09*</td>
<td>0.90 ± 0.09*</td>
<td>1.03 ± 0.09</td>
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Values are means ± SE; n = 8, 5, 7, and 4 mice for RyR2<sup>++/+</sup>/CNA-β<sup>++/+</sup>, RyR2<sup>++/+</sup>/CNA-β<sup>+/-</sup>, RyR2<sup>ADA/ADA</sup>/CNA-β<sup>++/+</sup>, and RyR2<sup>ADA/ADA</sup>/CNA-β<sup>+/-</sup> groups, respectively. *P < 0.05 compared with RyR2<sup>++/+</sup>/CNA-β<sup>++/+</sup>; †P < 0.05 compared with RyR2<sup>++/+</sup>/CNA-β<sup>+/-</sup>; ‡P < 0.05 compared with RyR2<sup>ADA/ADA</sup>/CNA-β<sup>++/+</sup>; §P < 0.05 compared with RyR2<sup>ADA/ADA</sup>/CNA-β<sup>+/-</sup>.
significantly upregulated in embryonic hearts and is not essential for cardiac hypertrophy in homozygous mice expressing a ryanodine receptor with mutations in the CaM binding domain that abrogate CaM binding. In addition, the studies suggest that class II HDAC/MEF2 signaling, another extensively studied signaling mechanism implicated in cardiac hypertrophy, is not upregulated in embryonic mutant hearts. In contrast, the data provide evidence that the Erk/p90RSK signaling pathway likely leading to reduced GSK-3β activity may have a role in promoting the development of cardiac hypertrophy in postnatal Ryr2^ADA/ADA^ mice.

Cardiac hypertrophy is a complex process involving multiple signaling pathways that appear to initiate during the embryonic life of Ryr2^ADA/ADA^ mice. Echocardiography indicated that chamber dilation of the left ventricle and decreased fractional shortening were less severe in 1-day-old compared with 10-day-old mice. Even though heart weight was unchanged between WT and Ryr2^ADA/ADA^ E16.5 mice, there was evidence for upregulation of genes and enzymatic activities associated with cardiac hypertrophy in embryonic Ryr2^ADA/ADA^ hearts. Thus early changes in gene expression in the embryonic heart may contribute to the rapid and progressive cardiac growth response in postnatal Ryr2^ADA/ADA^ mice.

To test for temporal changes in calcineurin and NFAT signaling, Ryr2^ADA/ADA^ mice were created that carried the luciferase transgene driven by the NFAT-dependent promoter. NFAT-dependent reporter activity is known to respond to stimuli that induce pathological hypertrophy, but not to stimuli associated with physiological cardiac growth (47). In a previous study, the hearts of transgenic mice carrying the luciferase gene driven by NFAT-dependent promoter had a maximal basal NFAT-luciferase activity at postnatal day 3 (47). We observed in hearts of E16.5 Ryr2^ADA/ADA^ mice a small increase in NFAT-luciferase activity, which was significantly upregulated in 1- and 10-day-old Ryr2^ADA/ADA^ mice. Thus an increased calcineurin signaling may have contributed to the rapid progression of cardiac hypertrophy in postnatal Ryr2^ADA/ADA^ mice.

Given that calcineurin/NFAT signaling was reported to be both necessary and sufficient for pathological cardiac hypertrophy (7), it was conceivable that Ryr2^ADA/ADA^ mice lacking...
CNA-β would show a reduced hypertrophic growth response. Earlier studies demonstrated that CNA-β null mice are viable and do not exhibit increased heart weight when subjected for 14 days to transaortic banding or infusion with angiotensin II or isoproterenol (7). Increases in mRNA and protein levels of CNA-β, but not CNA-α, in response to pressure overload or agonist stimulation, suggested a specific role for CNA-β in the hypertrophic growth response in hearts (42). The present study shows that ablation of CNA-β from Ryr2\textsuperscript{AD/ADA} mice improved overall cardiac function, albeit to a level less than WT. Moreover, ablation of CNA-β did not compensate for the reduced expression of RyR2 and SR Ca\textsuperscript{2+} pump-mediated Ca\textsuperscript{2+} uptake activity in 10-day-old Ryr2\textsuperscript{AD/ADA} hearts. The data suggest that calcineurin/NFAT signaling contributes but is not essential for the cardiac hypertrophic phenotype in Ryr2\textsuperscript{AD/ADA} mice.

Our results show that ablation of CNA-β from Ryr2\textsuperscript{AD/ADA} mice failed to reduce mRNA expression levels of genes implicated in cardiac hypertrophy such as β-MHC, ANP, and BNP. This finding is consistent with the reported elevated ANP levels in postnatal hearts, increased mRNA levels of β-MHC, increased Ca\textsuperscript{2+} pump, and protein kinases C and D over, no increase was observed in the in vitro phosphorylation and functional analyses have revealed an ANP regulatory role for Ca\textsuperscript{2+} pump-mediated Ca\textsuperscript{2+} uptake activity in 10-day-old Ryr2\textsuperscript{AD/ADA} hearts. The data suggest that calcineurin/NFAT signaling contributes but is not essential for the cardiac hypertrophic phenotype in Ryr2\textsuperscript{AD/ADA} mice.

We also note that to perform reporter measurements with the double targeted mice is independent of CNA-β. One possible explanation is a structural difference in their promoters. Structural elements (22, 44) suggested that the expression of CNA-β only marginally affects cardiac hypertrophy of Ryr2\textsuperscript{AD/ADA} mice. Further studies will be required to determine how a defective SR Ca\textsuperscript{2+} release regulates signaling pathways leading to the early phosphorylation and inhibition of GSK-3β in embryonic Ryr2\textsuperscript{AD/ADA} mice.

Embryonic hearts differ from adult hearts in gene expression profile, cell-cell interactions, and Ca\textsuperscript{2+} handling (25, 32, 36, 38, 39). Mutant mice lacking RyR2 die at approximately embryonic day 10 with morphological abnormalities in the heart tube (43). Functional RyR2s were detected in cardiomyocytes as early as embryonic days 9 to 10 (36, 38), supporting a role for RyR2 in embryonic heart. Thus a key feature of Ryr2\textsuperscript{AD/ADA} mice is that they are likely subjected to aberrant SR Ca\textsuperscript{2+} release at embryonic age earlier than 16.5 days. Neither CNA-β/NFAT nor HDAC4/MEF2 activities were substantially upregulated at embryonic day 16.5 in the mutant mice. Rather, our data suggest that the Erk/p90RSK signaling pathway likely leading to reduced GSK-3β activity may have a role in promoting the development of cardiac hypertrophy in postnatal Ryr2\textsuperscript{AD/ADA} mice. The identity of additional mechanisms linking impaired SR Ca\textsuperscript{2+} release to abnormal myocardial growth in Ryr2\textsuperscript{AD/ADA} mice remains to be established.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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