Ryanodine Receptor Type 2 Is Required for the Development of Pressure Overload-Induced Cardiac Hypertrophy

Yunzeng Zou, Yanyan Liang, Hui Gong, Ning Zhou, Hong Ma, Aili Guan, Aijun Sun, Ping Wang, Yuhong Niu, Hong Jiang, Hiroyuki Takano, Haruhiro Toko, Atsushi Yao, Hiroshi Takeshima, Hiroshi Akazawa, Ichiro Shiojima, Yuqi Wang, Isssei Komuro, Junbo Ge

Abstract—Ryanodine receptor type 2 (RyR-2) mediates Ca\(^{2+}\) release from sarcoplasmic reticulum and contributes to myocardial contractile function. However, the role of RyR-2 in the development of cardiac hypertrophy is not completely understood. Here, mice with or without reduction of RyR-2 gene (RyR-2\(^{-/-}\) and wild-type, respectively) were analyzed. At baseline, there was no difference in morphology of cardiomyocyte and heart and cardiac contractility between RyR-2\(^{-/-}\) and wild-type mice, although Ca\(^{2+}\) release from sarcoplasmic reticulum was impaired in isolated RyR-2\(^{-/-}\) cardiomyocytes. During a 3-week period of pressure overload, which was induced by constriction of transverse aorta, isolated RyR-2\(^{-/-}\) cardiomyocytes displayed more reduction of Ca\(^{2+}\) transient amplitude, rate of an increase in intracellular Ca\(^{2+}\) concentration during systole, and percentile of fractional shortening, and hearts of RyR-2\(^{-/-}\) mice displayed less compensated hypertrophy, fibrosis, and contractility; more apoptosis with less autophagy of cardiomyocytes; and similar decrease of angiogenesis as compared with wild-type ones. Moreover, constriction of transverse aorta-induced increases in the activation of calcineurin, extracellular signal-regulated protein kinases, and protein kinase B/Akt but not that of Ca\(^{2+}\)/calmodulin-dependent protein kinase II, and its downstream targets in the heart of wild-type mice were abolished in the RyR-2\(^{-/-}\) one, suggesting that RyR-2 is a regulator of calcineurin, extracellular signal-regulated protein kinases, and Akt but not of calmodulin-dependent protein kinase II activation during pressure overload. Taken together, our data indicate that RyR-2 contributes to the development of cardiac hypertrophy and adaptation of cardiac function during pressure overload through regulation of the sarcoplasmic reticulum Ca\(^{2+}\) release; activation of calcineurin, extracellular signal-regulated protein kinases, and Akt; and cardiomyocyte survival.

Key Words: Ca\(^{2+}\) • calcineurin • cardiac hypertrophy • pressure overload • ryanodine receptor

Cardiac hypertrophy is usually a compensatory response of the heart to hemodynamic overload, including hypertension, valve diseases, and myocardial infarction. At the beginning, cardiac hypertrophy has beneficial effects on maintaining cardiac output by reducing wall stress. Prolonged hypertrophy, however, may eventually lead to heart failure (HF). Development of cardiac hypertrophy and HF is a complex process involving many aspects of alterations, such as abnormality of intracellular Ca\(^{2+}\) homeostasis, activation of a variety of protein kinases, reprogramming of specific gene expression, disorders of metabolism, and so forth. Among these alterations, abnormality of intracellular Ca\(^{2+}\) homeostasis is a critical one.

Intracellular Ca\(^{2+}\) homeostasis is regulated by Ca\(^{2+}\) handling proteins, which control cardiomyocyte function, including excitation-contraction (E-C) coupling of cardiomyocytes. Voltage-dependent L-type Ca\(^{2+}\) channels (LCC) activated by membrane depolarization allow Ca\(^{2+}\) entry into the cell, triggering a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from sarcoplasmic reticulum (SR) through a ryanodine type 2 receptor (RyR-2) and muscle contraction. Elevated cytosolic Ca\(^{2+}\) is then removed from the cytosol for relaxation, which...
is mediated by several pathways involving uptake to SR by SR Ca\(^{2+}\)-ATPase 2 (SERCA2), uptake to mitochondria by Ca\(^{2+}\) uniport, and extrusion from the cell by Na\(^{+}/Ca^{2+}\) exchanger (NCX). \(^7\)

Alterations in Ca\(^{2+}\) handling proteins, such as RyR-2, LCC, SERCA2, and NCX, not only induce abnormality of CICR but also associate with cardiac hypertrophy and HF. \(^7\)–\(^15\) Because RyR-2 is a major CICR channel, it might greatly regulate the development of cardiac hypertrophy. FK506-binding protein (FKBP) 12.6 is a regulatory protein that tightly binds to RyR-2 and stabilizes the channel. \(^16\)–\(^17\) Numerous lines of clinical and experimental evidence have indicated that altered RyR-2 gene and function contribute to cardiac disorders, including catecholaminergic polymorphic ventricular tachycardia or arrhythmogenic right ventricular cardiomyopathy type 2, \(^15\) cardiac hypertrophy, and dysfunction. \(^18\)–\(^19\)

Furthermore, RyR-2 is hyperphosphorylated by protein kinase A (PKA) in HF, resulting in dissociation of FKBP12.6 from RyR-2 and Ca\(^{2+}\) leaks from SR in diastole, which impair contractility. \(^16\)–\(^17\) Thus, alteration in RyR-2 is critically involved in abnormality of Ca\(^{2+}\) handling and contractile dysfunction in HF. However, the role of RyR-2 in the development of cardiac hypertrophy, especially in the pressure overload-induced one, is still not completely understood.

Moreover, because intracellular Ca\(^{2+}\) concentration cyclically alters during each heartbeat, it is assumed that there exist specialized microdomains where Ca\(^{2+}\) functions as a signaling molecule independent of E-C coupling. \(^20\) How Ca\(^{2+}\) enters into these potential microdomains in response to hypertrophic stimuli remains unknown, although inositol 1,4,5-triphosphate receptors, T-type Ca\(^{2+}\) channels, and transient receptor potential channels have been suggested as possible sources of E-C coupling-independent Ca\(^{2+}\) entry. \(^21\)

In addition, Ca\(^{2+}\) activates 2 major Ca\(^{2+}\)-dependent hypertrophic signaling pathways, the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII)-histone deacetylase (HDAC) pathway and the calcineurin (CnA)-nuclear factor of activated T cells (NFAT) pathway. CaMKII induces phosphorylation and nuclear export of HDAC, leading to the relief of HDAC-mediated transcriptional repression of myocyte enhancer factor 2 (MEF2) and MEF2-dependent transcription of hypertrophic genes. \(^22\) CnA dephosphorylates NFAT, which leads to nuclear translocation of NFAT and upregulation of NFAT-dependent transcription of hypertrophic genes such as atrial natriuretic peptide (ANP) and GATA binding protein 4 (GATA4). \(^23\) Although CaMKII has been indicated to phosphorylate and activate RyR-2, \(^24\) there is no direct evidence showing the relationship between RyR-2 and CaMKII or CnA, especially during the development of cardiac hypertrophy after pressure overload.

Additionally, we have reported previously that CnA regulates cardiomyocyte hypertrophy induced by isoproterenol through activation of extracellular signal-regulated kinases (ERKs), a critical regulator of cell growth. \(^25\) Protein kinase B/Akt is also known to be upregulated in response to pressure overload and implicated in the development of load-induced cardiac hypertrophy. The present study was, therefore, designed to elucidate the effects of RyR-2 reduction on the development of hemodynamic overload-induced cardiac hypertrophy and the relative mechanisms.

### Methods

An expanded Methods section is available in the online Data Supplement (please see http://hyper.ahajournals.org).

### Mice

Generation of RyR-2 mutant mice (C57Bl/6 strain), identification of the mutation, and induction of pressure overload in 12-week-old male mice by thoracic aorta constriction (TAC) have been described previously. \(^24\)–\(^26\)–\(^27\) The mice were housed in a room with a 12-hour light/dark cycle and allowed free access to food and water. All of the animal protocols were approved by the Fudan University Animal Care and Use Committee and complied with Guidelines for the Care and Use of Laboratory Animals published by the National Academy Press (National Institutes of Health Publication No. 85-23, 1996).

### Echocardiography and Catheterization Measurement

Transthoracic echocardiography was performed by using an animal-specific instrument (Visual Sonics Vevo770, Visual Sonics Inc). \(^27\)–\(^28\) Mice were anesthetized with isoflurane, and M-mode images of the left ventricle (LV) were recorded when the heart rate of the mice was maintained at 450 to 500 bpm by changing isoflurane concentrations from 0.5% to 4.0% according to heart rate. \(^28\) Hemodynamic parameters such as blood pressure (BP) and left ventricular (LV) end-diastolic pressure were measured by a 1.4 F cardiac catheter (Millar Instruments, Inc) connected to a Mac laboratory system (AD Instruments).

### Isolation of Adult Cardiomyocytes and Intracellular Ca\(^{2+}\) Measurement

Adult cardiomyocytes were isolated from mice by Langendorff perfusion method, and intracellular Ca\(^{2+}\) concentration was measured by use of fluo-3, as described previously. \(^29\)

### Histological Analyses

Cardiomyocyte hypertrophy and extent of LV fibrosis were measured in hematoxylin and eosin and van Gieson stained LV sections, respectively. \(^27\)–\(^30\) The density of capillaries in LV section was measured by use of fluorescein isothiocyanate-labeled peanut agglutinin and the relative mechanisms.

### Statistical Analysis

All of the values are expressed as mean±SD of >3 experiments. Values of \(P<0.05\) were considered statistically significant.
Results

Altered Ca\(^{2+}\) Handling in Adult Cardiomyocytes of RyR-2\(^{-/-}\) Mice

We first examined the impact of heterozygous disruption of the RyR-2 gene on Ca\(^{2+}\) handling and contractility in isolated adult cardiomyocytes (Table S1, please see http://hyper.ahajournals.org). At basal condition, Ca\(^{2+}\) transient amplitude, the rate of an increase in intracellular Ca\(^{2+}\) concentration during systole, and percentile of fractional shortening were significantly reduced in RyR-2\(^{-/-}\) cardiomyocytes compared with wild-type (WT) ones, although the half-time of relaxation from caffeine-induced contracture (half-time of diastole phase) was comparable between them. At 3 weeks after TAC, Ca\(^{2+}\) transient amplitude, rate of an increase in intracellular Ca\(^{2+}\) concentration during systole of systolic phase, and percentile of fractional shortening were significantly reduced both in WT and RyR-2\(^{-/-}\) cardiomyocytes, whereas half-time of diastole phase was extended in WT but not in RyR-2\(^{-/-}\) cardiomyocytes. However, the reduction of Ca\(^{2+}\) transient amplitude, rate of an increase in intracellular Ca\(^{2+}\) concentration during systole, and percentile of fractional shortening after TAC in RyR-2\(^{-/-}\) cardiomyocytes were more than in WT ones. On the other hand, the addition of ryanodine resulted in a significant decrease of the percentile of fractional shortening in WT cardiomyocytes but only a slight reduction in RyR-2\(^{-/-}\) cardiomyocytes (Table S2).

Attenuated Cardiac Hypertrophy and Contractility in RyR-2\(^{-/-}\) Mice in Response to Pressure Overload

There was no significant difference in cardiac dimension and contractility evaluated by echocardiography between sham-operated WT and RyR-2\(^{-/-}\) mice (Figure 1 and Table). At 2 days after TAC, a time point without efficient compensation of LV hypertrophy under hemodynamic overload, although there was a slight difference in cardiac hypertrophy between the 2 types of mice, LV dysfunctions characterized by LV ejection fraction, LV fractional shortening, LV end-diastolic pressure, and LV pressure increase and decay (dP/dt) were significantly impaired in RyR-2\(^{-/-}\) mice compared with WT mice. At 3 weeks after TAC, thickness of interventricular septum in diastole and LV contractility in RyR-2\(^{-/-}\) mice were both significantly reduced compared with those in WT mice, and hemodynamic studies showed the increased LV end-diastolic pressure and decreased peak dP/dt (dP/dt\(_{\text{max}}\)) in RyR-2\(^{-/-}\) mice compared with WT mice. Heart weight/body weight ratio of RyR-2\(^{-/-}\) mice 3 weeks after TAC was significantly smaller than that of WT mice (Table), and histological analysis revealed that heart size and cross-sectional area of cardiomyocytes were significantly reduced in RyR-2\(^{-/-}\) mice compared with WT mice (Figure 2A and 2B). The examination of isolated cardiomyocytes also confirmed that the surface area of isolated cardiomyocytes was significantly reduced in RyR-2\(^{-/-}\) cells compared with WT.
cells after TAC, although they were comparable between the 2 types of cells at baseline (Figure 2C). The extent of myocardial fibrosis evaluated by histology, Western blot analysis for collagen Iα expression in LV tissue, and growth of isolated cardiac fibroblasts was significantly reduced in RyR-2+/− mice compared with WT mice (Figure 3). There was no significant difference in these parameters between WT and RyR-2+/− mice at baseline.

**Alteration of Hemodynamic Load-Responsive Gene Expression Program**

Induction of fetal-type cardiac genes is one of the hypertrophic responses of the heart. We examined the expression of ANP, brain natriuretic peptide (BNP), and skeletal α-actin (α-SKA) genes in the heart after TAC. TAC-mediated induction of ANP and BNP genes was downregulated, whereas induction of the α-SKA gene was not altered at day 2 and enhanced 3 weeks after TAC in the heart of RyR-2+/− mice (Figure 4).

The expression of genes encoding Ca2+ handling proteins is also responsive to hemodynamic load. We, therefore, examined the expression of SERCA2, LCC, and NCX genes, as well as RyR-2 genes, in the heart after TAC. In WT hearts, 3 weeks of pressure overload induced downregulation of RyR-2 and SERCA2 genes and upregulation of LCC and NCX genes. However, in RyR-2+/− hearts, the expression levels of SERCA2 and LCC genes were not altered, whereas the NCX gene was downregulated (Figure 5A through 5E).

We also assessed the effects of the reduction of the RyR-2 gene on protein expression and phosphorylation state of the receptor. The changes of RyR-2 protein were similar to those of the RyR-2 gene (Figure 5F). RyR-2 function is affected by its binding proteins, such as FKBP 12.6 and PKA.15-17,21,24 We, thus, examined the binding of these 2 proteins with RyR-2 in WT and RyR-2+/− mice (Figure S1). The amount of FKBP 12.6-associated RyR-2 was less in the RyR-2+/− heart compared with the WT, whereas PKA-phosphorylated RyR-2 in the RyR-2+/− heart was more than the WT heart under basal condition, suggesting that an upregulation of RyR-2 phosphorylation compensated for RyR-2 deficiency. TAC for 3 weeks decreased the amount of FKBP 12.6-associated RyR-2 not only in WT mice but also in RyR-2+/− mice. However, TAC-increased PKA phosphorylation of RyR-2 in the RyR-2+/− heart was more than in the WT heart.

**Attenuated Activation of Hypertrophic Signaling Pathways in Response to Pressure Overload in RyR-2+/− Mice**

Because pressure overload-induced cardiac hypertrophy is attenuated in RyR-2+/− mice, we examined whether the load-induced activation of hypertrophic signaling pathways31 is impaired in RyR-2+/− hearts. CaMKII and CnA are critical regulators of Ca2+-mediated cardiac hypertrophy, and pressure overload activated these 2 proteins (Figure 6A and 6B) and their respective target molecules, HDACs/MEF2 and GATA4 (Figure S2A and S2B) in WT hearts. However, activation of CnA and expression of GATA4 mRNA in response to pressure overload were impaired in RyR-2+/− hearts, whereas the activation of CaMKII and HDACs and expression of the MEF2 gene were comparable between WT and RyR-2+/− hearts. To ask whether the changes of activation of CaMKII and CnA were because of the changes in their protein expression, we examined the expression of total CaMKII and CnA proteins in LV tissues (Figure 6A and 6B). Although CaMKII protein was only slightly increased in both types of mice at 3 weeks after TAC, CnA protein was similarly increased in the 2 mice, suggesting that the increases in cardiac CaMKII phosphorylation in both types of mice and the decrease of CnA phosphorylation in RyR-2+/− mice after 3 weeks of TAC are not because of the changes of CaMKII and CnA protein expression. Signaling pathways mediated by ERK1/2 and Akt are also known to be upregulated in response to pressure overload and implicated in the development of load-induced cardiac hyper-

### Table. Biometric, Echocardiographic, and Hemodynamic Parameters at 2 d and 3 wk After Sham and TAC Operation

<table>
<thead>
<tr>
<th>Mice TAC</th>
<th>Sham-2d</th>
<th>TAC-2d</th>
<th>Sham-3W</th>
<th>TAC-3W</th>
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<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>BP, mm Hg</td>
<td>93.0±11.0</td>
<td>153.0±14.0*</td>
<td>90.0±12.0</td>
<td>156.0±18.0*</td>
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<tr>
<td>BW, g</td>
<td>23.4±2.8</td>
<td>23.9±2.2</td>
<td>23.8±2.4</td>
<td>24.0±2.3</td>
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<tr>
<td>HW, mg</td>
<td>112.5±10.8</td>
<td>135.0±9.3*</td>
<td>118.3±15.1</td>
<td>227.2±37.3*</td>
</tr>
<tr>
<td>BW/BW, mm/g</td>
<td>4.8±2.1</td>
<td>5.8±1.0*</td>
<td>4.9±0.8</td>
<td>9.1±1.3*</td>
</tr>
<tr>
<td>LVWs, mm</td>
<td>0.75±0.35</td>
<td>1.55±0.42*</td>
<td>1.77±0.32</td>
<td>1.85±0.35</td>
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<tr>
<td>LVWd, mm</td>
<td>3.38±0.45</td>
<td>3.40±0.32</td>
<td>3.43±0.35</td>
<td>3.96±0.52</td>
</tr>
<tr>
<td>LVePD, mm Hg</td>
<td>3.5±0.6</td>
<td>3.0±1.5</td>
<td>4.0±0.5</td>
<td>7.2±2.0*</td>
</tr>
<tr>
<td>dP/dtmax, mm Hg</td>
<td>2120±300</td>
<td>2670±310</td>
<td>2140±160</td>
<td>4090±250*</td>
</tr>
<tr>
<td>dP/dtmin, mm Hg</td>
<td>2060±320</td>
<td>2150±260†</td>
<td>2100±300</td>
<td>2230±347†</td>
</tr>
<tr>
<td>LPDc, mm Hg</td>
<td>2010±280</td>
<td>2520±250*</td>
<td>2070±90</td>
<td>3750±320*</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>0.8±9.1</td>
<td>3.2±0.45</td>
<td>3.25±0.45</td>
<td>3.81±0.31*</td>
</tr>
</tbody>
</table>

*P<0.05 vs respective sham.
†P<0.05 vs corresponding WT with TAC.
trophy. TAC for 3 weeks induced robust activation of ERK1/2 and Akt in WT hearts but not in RyR-2+/− hearts (Figure 6C and 6D). Also, activities of p70S6 kinase, a downstream molecule of Akt1, were consistent with phosphorylation of Akt1 (Figure S3).

Increased Apoptosis, Decreased Autophagy, and Unchanged Angiogenesis in Pressure-Overloaded Heart

Although heterozygous deletion of the RyR-2 gene led to abnormal Ca2+ homeostasis and contractility in isolated cardiomyocytes, contractile function in vivo was not altered in RyR-2+/− mice, indicating that the alteration in Ca2+ handling is compensated in in vivo situation. Nonetheless, contractile function was significantly reduced in RyR-2+/− mice compared with WT mice after pressure overload. This suggests that there exist additional mechanisms that contribute to impaired cardiac function in RyR-2+/− mice independent of the alteration in Ca2+ homeostasis.

Apoptosis of cardiomyocytes is a well-established mediator of HF. Activity of caspase-3 and cleaved poly (ADP-ribose) polymerase 1, markers of apoptosis, were significantly increased in RyR-2+/− hearts compared with the WT heart (Figure 7A). The amount of cleaved poly (ADP-ribose) polymerase 1, another marker of apoptosis, was also increased in the RyR-2+/− heart compared with the WT heart (Figure 7B). Thus, heterozygous deletion of the RyR-2 gene led to increased apoptosis of cardiomyocytes after chronic pressure overload.
Although the role of autophagy in the pathogenesis of HF is controversial, autophagy of cardiomyocytes appears to be an adaptive response to the increased cardiac workload, at least in some situations.\textsuperscript{33,34} Immunostaining and Western blot analysis revealed that the extent of autophagy after pressure overload, as evidenced by LC3b-positive dots or increased expression of beclin1, was attenuated in \textit{RyR-2}\textsuperscript{+/+}\textit{/H11001}\textit{/H11002} hearts compared with WT hearts (Figure 7C and 7D), indicating that heterozygous deletion of the \textit{RyR-2} gene led to decreased autophagy in the heart after pressure overload.

Myocardial ischemia induced by impaired coronary angiogenesis is another critical mediator of contractile dysfunction.\textsuperscript{27,35,36} In the present study, however, the number of capillaries and the expression of vascular endothelial growth factor were not altered between WT and \textit{RyR-2}\textsuperscript{+/+}\textit{/H11001}\textit{/H11002} hearts after chronic pressure overload (Figure 8), suggesting that myocardial ischemia does not specifically contribute to contractile dysfunction in \textit{RyR-2}\textsuperscript{+/+}\textit{/H11001}\textit{/H11002} hearts.

**Figure 3.** Analyses for myocardial fibrosis 3 weeks after sham or thoracic aorta constriction (TAC). A, Representative photomicrographs of van-Gieson–stained left ventricular (LV) sections. Fibrosis area was measured in 8 fields from 1 LV section and 5 sections from 1 heart. B and C, Western blot analysis for collagen 1\textalpha{} protein expression. GAPDH was served as a loading control. The intensities of collagen 1\textalpha{} were quantitated as folds of GAPDH. Data represent mean±SD from 6 hearts. D, DNA synthesis in isolated cardiac fibroblasts. DNA synthesis was evaluated by measuring the incorporation of [\textsuperscript{3}H]thymidine into the cultured fibroblasts. Data represent mean±SD from 3 mice. *\textit{P}<0.05 vs respective sham; †\textit{P}<0.05 vs corresponding WT-TAC.

Discussion

\textit{RyR-2} mediates \textsuperscript{2+}Ca release from the SR and plays a central role in the regulation of \textsuperscript{2+}Ca homeostasis and contractility in cardiac myocytes.\textsuperscript{6,17} In the present study, we have demonstrated that \textit{RyR-2} also plays a critical role in the development of cardiac hypertrophy and hypertrophic responses of the heart.

It was reported previously that homozygous deletion of the \textit{RyR-2} gene resulted in embryonic lethality at approximately embryonic day 10, with morphological abnormalities of the heart.\textsuperscript{16} In \textit{RyR-2}\textsuperscript{+/+}\textit{/H11001}\textit{/H11002} mice, there was no apparent cardiac phenotype at baseline, as evidenced by echocardiography, hemodynamic studies, and histological analyses. However, abnormal \textsuperscript{2+}Ca release from the SR and reduced contractility were observed in isolated adult cardiomyocytes obtained from \textit{RyR-2}\textsuperscript{+/+}\textit{/H11001}\textit{/H11002} mice. This indicates that a defect in \textsuperscript{2+}Ca handling at the single cell level is compensated in \textit{RyR-2}\textsuperscript{+/+}\textit{/H11001}\textit{/H11002} mice at the organ level. The expression levels of \textsuperscript{2+}Ca...
handling proteins (SERCA2, LCC, and NCX) other than RyR-2 were not altered between WT and RyR-2/H11001/H11002 mice at baseline. Although the exact nature of this compensation is unknown at present, there are 2 possibilities that might contribute to the compensation. At first, activation of the sympathetic nervous system because of the decrease of RyR-2 might enhance the in vivo cardiac performance at baseline. The second could be attributed to the in vivo hyperphosphorylation and upregulation of the function of RyR-2 because of the decrease of the receptor. Our data showing the increases in association of RyR-2 with PKA and dissociation of FKBP12.6 from the receptor in the heart of RyR-2/H11001/H11002 mice at baseline provide evidence for this explanation.

It has been known that RyR-2 is downregulated and hyperphosphorylated in HF.17 We also observed that the degree of downregulation of the RyR-2 gene and protein was associated with the degree of heart dysfunction in mice. Furthermore, we have shown that RyR-2+/− mice exhibited impaired contractility in response to pressure overload both at the early and late stages, indicating that RyR-2 is required for the maintenance of contractile function under hemodynamic stress. One explanation for this is that a compensatory mechanism that maintains the contractile function of RyR-2+/− heart in vivo is disrupted by extensive overload, leading to the impairment of contractile function in RyR-2+/− mice under hemodynamic stress. An alternative and not mutually exclusive explanation is that there exist additional mechanisms that contribute to the contractile dysfunction of RyR-2+/− mice independent of the alteration in Ca2+ handling. One possible Ca2+-independent mechanism of contractile dysfunction is cardiomyocyte apoptosis. TUNEL-positive apoptotic myocytes were increased in the heart of RyR-2+/− mice compared with WT mice after chronic pressure overload. It has also been indicated that pressure overload-induced cardiac hypertrophy is initiated by a wave of apoptosis of cardiomyocytes, which is involved in the pathogenesis of cardiac remodelling.37 Because cardiomyocyte apoptosis plays a causal role in contractile dysfunction and HF,27,32 increased apoptosis of cardiomyocytes may be a potential mechanism of impaired contractility rather than induction of hypertrophy in RyR-2+/− mice under hemodynamic overload in the setting. Although the exact mechanism by which apoptosis is increased in the heart of RyR-2+/− mice is not clear, it may be because of an indirect mechanism involving attenuated activation of cell survival pathways, such as ERK and Akt. Another possible Ca2+-independent mechanism of contractile dysfunction is autophagy, and the extent of autophagy in response to pressure overload was decreased in the heart of RyR-2+/− mice compared with WT mice. Autophagy is a highly conserved cellular mechanism of protein recycling that can lead to cell survival or death. The causal role of autophagy in the pathogenesis of cardiac remodeling and HF is controversial,33,34 and reduced autophagy may simply reflect the attenuated cardiac hypertrophy and impaired cardiac function in the RyR-2+/− heart. The
third possible Ca\(^{2+}\)-independent mechanism of contractile dysfunction is myocardial ischemia.\(^{35,36}\) It was shown that impaired coronary angiogenesis in the chronic stage of pressure overload contributes to the transition from adaptive to maladaptive cardiac hypertrophy.\(^{27}\) However, coronary vessel number and the expression of vascular endothelial growth factor in the heart were not altered between WT and RyR-2\(^{-/-}\) mice, suggesting that this mechanism does not contribute to the impaired contractility of the RyR-2\(^{-/-}\) heart under chronic overload.

In this study we have also shown that RyR-2\(^{-/-}\) mice exhibited attenuated cardiac hypertrophy and myocardial fibrosis in response to pressure overload. These were associated with reduced or altered hypertrophic responses of the heart. Van Oort et al\(^{19}\) have reported recently that, in RyR-2-R176Q knockin mice with the defective cardiac RyR-2, pressure overload induced an enhanced hypertrophic response compared with WT mice, which is attributed to an increased SR Ca\(^{2+}\) leak and the prohypertrophic CnA/NFAT pathway. In our present study, we observed decreased phosphorylation of CnA in the RyR-2\(^{-/-}\) heart compared with WT mice under the condition of pressure overload. Although the reason for this difference is not clearly understood, there are several possibilities. At first, mice models are different. We used heterozygous RyR-2-knockout mice, whereas Van Oort et al\(^{19}\) used RyR-2-R176Q knockin mice. In RyR-2\(^{-/-}\) mice, although the expression of RyR-2 is about half decreased and the remained RyR-2 channels are hyperphosphorylated at
basal condition, the structural, functional, and hemodynamic abnormalities of the heart are not found. In RyR-2-R176Q knockin mice, although the structures are normal, there are lower end-diastolic volume and higher end-diastolic pressure compared with WT mice. These differences between the 2 types of mice might contribute to the different results of cardiac hypertrophy. Secondly, the period after TAC is different. We observed pressure overload-induced cardiac hypertrophy within 3 weeks, whereas Van Oort et al showed the results at 8 weeks after TAC. The third one is SR Ca2+/H11001 leak and activation of CnA. Van Oort et al have demonstrated the increases in SR Ca2+/H11001 leak and activation of the CnA pathway in RyR-2-R176Q knockin mice under TAC condition, whereas we observed a decrease of SR Ca2+ release and CnA phosphorylation after TAC in RyR-2+/− mice.

Of the 2 major regulators of Ca2+-mediated cardiac hypertrophy, CaMKII activation, and its target molecules, HDACs and MEF2 were comparable between WT and RyR-2+/− mice, whereas CnA activation and its target GATA4 were severely attenuated in the heart of RyR-2+/− mice compared with those in WT mice. We have shown previously that CnA activation requires a potent CICR in cardiomyocytes, whereas CaMKII could be activated without increase in intracellular Ca2+ (Figure S4), suggesting the possibility that, although reduced CICR in RyR-2+/− cardiomyocytes does...
expression and/or localization of potential mediators of E-C coupling-independent Ca\(^{2+}\) entry, leading to impaired activation of Ca\(^{2+}\)-dependent hypertrophic signaling pathways. Additionally, chronic pressure overload-induced ERK1/2 and Akt activation was also attenuated in the heart of RyR-2\(^{-/-}\) mice, although the exact mechanisms of downregulation of these signaling pathways are not clear. We reported previously that ERK activation in cardiomyocytes by isoproterenol is mediated by CnA,

arguing against the possibility that attenuated CnA activation contributes to impaired ERK activation, our results suggest that activation of ERKs and Akt by pressure overload, at least in part, requires RyR-2-dependent activation of CnA, which associates with myocardial hypertrophy and fibrosis.

We conclude that RyR-2 contributes to the development of cardiac hypertrophy and adaptation of cardiac function during pressure overload through regulation of Ca\(^{2+}\) handling. Van Oort et al\(^{19}\) have demonstrated that the increased Ca\(^{2+}\) release through RyR-2 from the SR induces activation of CnA and dephosphorylation and translocation of NFAT3 into the nucleus, thereby inducing hypertrophic gene expression.\(^{19}\) We speculate here that, other than activation of the CnA/NFAT3 pathway, RyR-2-mediated Ca\(^{2+}\) release from the SR induces activation of the CnA/ERKs, Akt, and cardiomyocyte survival pathway, which may also contribute to pressure overload-induced cardiac hypertrophy. Activation of CnA/KII and myocardial angiogenesis seems to be not affected by RyR-2 deficiency.

**Perspectives**

By using the RyR-2-deficient mice, we have provided direct evidence to demonstrate that, in addition to its
well-established role in the regulation of E-C coupling, RyR-2 also regulates cardiac hypertrophy in response to pressure overload. Further elucidation of the exact mechanisms of RyR-2-mediated cardiac hypertrophy will lead to the in-depth understanding of cardiac hypertrophy and HF.

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Disclosures
None.

References
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Ryanodine receptor type 2 is required for the development of pressure overload-induced cardiac hypertrophy

Yunzeng Zou 1, Yanyan Liang 1, Hui Gong 1, Ning Zhou 1, Hong Ma 1,7, Aili Guan 1, Aijun Sun 1, Ping Wang 2, Yuhong Niu 1, Hong Jiang 1, Hiroyuki Takano 2, Haruhiro Toko 2, Atsushi Yao 3, Hiroshi Takeshima 4, Hiroshi Akazawa 5, Ichiro Shiojima 5, Yuqi Wang 6, Issei Komuro 5, Junbo Ge 1

1 Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital and Institutes of Biomedical Sciences, Fudan University, Shanghai, China;
2 Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan
3 Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Tokyo, Japan;
4 Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan
5 Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan;
6 Department of Vascular Surgery, Zhongshan Hospital, Fudan University, Shanghai, China
7 Department of Cardiology, Second Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou, China,

*Correspondence to:
Yunzeng Zou, MD, PhD,
Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital and Institutes of Biomedical Sciences, Fudan University,
180 Feng Lin Road, Shanghai 200032, China,
TEL/FAX: +86-21-5423-7969;
Email: zou.yunzeng@zs-hospital.sh.cn


**Expanded Online Methods**

**Pressure Overload Model and**

Induction of pressure overload in 12-week-old male mice by thoracic aorta constriction (TAC) has been previously described. ¹ In brief, after anesthetization and artificial ventilation, the transverse aorta of mice was constricted with the 7-0 nylon suture by ligating the aorta together with a blunted 27-gauge needle, which was pulled out later. Hemodynamic parameters were measured by a 1.4 F Millar Micro-Tip Catheter Pressure Transducer (Millar Instruments, Inc.) inserted from the right carotid artery into aorta and LV. The transducer was connected to a Mac Lab system (AD Instruments) and the systolic blood pressure (BP) and LV end-diastolic pressure (LVEDP) were recorded.

**Isolation of Adult Cardiomyocytes and Intracellular Ca²⁺ Measurement**

Adult cardiomyocytes were isolated from mice by Langendorff perfusion method and intracellular Ca²⁺ concentration was measured as previously described. ² Briefly, dissected hearts were rapidly attached to a Langendorff perfusion system, perfused with Ca²⁺-free solution and digested with type II collagenase, protease and BDM in 100 mmol/L Ca²⁺ solution at 37°C for 8-12 min. Then, the hearts were washed and LV was excised, minced and shaken in 100 mmol/L Ca²⁺ solution. Cell suspension was filtered and isolated myocytes were kept in 1 mmol/L Ca²⁺ solution at room temperature (RT). Intracellular Ca²⁺ concentration was measured as previously described. ² In briefly, isolated myocytes were incubated with 3-4 μmol/L fluo-3 AM in HEPES solution for 30 min at RT. After washed by dye-free HEPES solution for more than 15 min, the cells were excited by UV light of 485 nm, and the emission of 530 nm was collected. Measurements were performed at RT within 6 hours after the isolation. The intracellular Ca²⁺ transients were electrically activated at 0.25 Hz, stabilized within 2 min, and then 10 mmol/L caffeine was rapidly applied to the cardiomyocytes just after the pacing was turned off. ²

**Cardiomyocyte Hypertrophy and Extent of Fibrosis in LV Tissues**

Heart sections were stained with hematoxylin and eosin (H-E) and cardiomyocyte hypertrophy was evaluated by measuring the cross section area of cardiomyocytes in randomly selected 10 points per LV section and 100 myocytes was calculated for one point. The value was expressed as the area of per 100 cardiomyocytes. The extent of LV fibrosis was measured in 8 fields randomly selected from a section by calculating the relative ratio of van Gieson-stained fibrosis area. ¹ ³

**Angiogenesis, Apoptosis and Autophagy in LV Section**

The density of capillaries in LV section was examined by CD31 immunostaining (PharMingen), and the number of CD31-positive microvessels per 100 cells was calculated. ² Apoptosis of cardiomyocytes was detected in situ by terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) kit (TaKaRa Biomedicals) in paraffin-embedded heart tissue sections. ¹ Autophagy of cardiomyocytes was evaluated by LC3b immunostaining using an anti–LC3b antibody (Santa Cruz Biotechnology Inc.) on paraffin sections. Co-staining of apoptosis and autophagy in LV section were performed by staining against a cardiomyocyte-specific antigen, α-MHC (Santa Cruz Biotechnology Inc.).

**Isolation and Culture of Adult Mouse Cardiac Fibroblasts**

LV tissues from adult mice with sham or TAC for 4 weeks were minced and digested in DMEM containing 0.05% Bovine Serum Albumin (BSA), 1000U/mL Collagenase 2 (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.003% trypsin at 37°C
with continuous shaking for 90 min. The dissociated cells were plated for 1 hour to allow fibroblasts to adhere. Following removal of non-adherent cells, cardiac fibroblasts were cultured to confluence in DMEM containing 2.5% FBS. Experiments were performed on the second passage cells plated at a density of ~200 cells per mm². The culture medium was changed to serum-free DMEM at 48 hours before use.

[^3]H|Thymidine Incorporation

DNA synthesis in cardiac fibroblasts was assessed by measuring[^3]H|thymidine incorporation into the cells. In brief, cultured cardiac fibroblasts were serum-deprived for 48 hours. [^3]H|thymidine (1.25 μCi/mL) was added 2 hours before the harvest. Plates were then placed on ice, quickly washed three times with 1 mL of ice-cold PBS, incubated 10 min with 1 mL of 10% trichloroacetic acid, and washed twice with 1 mL of 10% trichloroacetic acid and three times with 1 mL of 95% ethanol. Precipitates were solubilized for 1.5 hours in 800 μL of 0.2N NaOH and neutralized, and radioactivity was measured by liquid scintillation spectroscopy.

Western Blotting

Total protein (100 μg) of the LV tissue was size fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore). Antibodies used for immunoblotting were from Santa Cruz Biotechnology Inc. Phosphorylation sites recognized by anti phosphor Ark1 and CaMKII antibodies are Ser473 and Thr286, respectively. For detection of phosphorylation of CnA, total protein (500 μg) of the LV tissue was immunoprecipitated by an anti CnA antibody. The immunocomplexes were subjected to SDS-PAGE and blotted by an anti phosphor Ser antibody. The immunoreactivity was detected using an enhanced chemiluminescence reaction system (Amersham Pharmacia Biotech).

Detection of RyR-2 Protein Phosphorylation.

Cardiac SR was prepared from total LV lysates by centrifugation. Total proteins from cardiac SR (200 μg) were incubated with an anti RyR-2 antibody (Santa Cruz Biotechnology Inc.) and the immunocomplexes were subjected to non-reducing gel electrophoresis. For RyR-2 phosphorylation with PKA, protein A sepharose beads were incubated with PKA catalytic subunit before gel fraction. The membranes were immunoblotted with antibodies against RyR-2, FKBP12.6 or PKA (Santa Cruz Biotechnology Inc.).

p70 S6 Kinase Activity

LV tissues were lysed with 0.15 mL of Triton X-100 lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L EDTA, 40 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L leupeptin, 1 μmol/L pepstatin A, 1% Triton X-100) for 25 min on ice. Lysates (100 μg) were incubated for 12 hours at 4°C with anti-p70[^6]S6 kinase polyclonal antibody (Santa Cruz Biotechnology Inc.) preabsorbed to protein A-Sepharose beads. The immune complexes were washed 3 × with lysis buffer and once with kinase buffer (20 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4, 10 mmol/L MgC12, 1 mmol/L dithiothreitol (DTT), 10 mmol/L β-glycerophosphate). The immunoprecipitates were resuspended in 40 μL of kinase buffer containing 0.2 mmol/L S6 peptide (RRRLSSLRA, Santa Cruz Biotechnology Inc.), 20 μmol/L ATP and 5 μCi of [γ-32p]ATP (3000 Ci/mmoll and incubated at 30°C for 20 min. After incubation, the reactions were stopped by spotting the mixture on P81 paper (Whatman). The filters were washed 3 × for 10 min each in 1% phosphoric acid, once in acetone and dried. The 32p uptake was measured by Cerenkov counting method.
RT-PCR Analysis
Total RNA was isolated from LV tissues using TRIzol reagent (Gibco BRL). The expression of GATA4 and MEF2C at the mRNA levels was evaluated using RT-PCR. The primers were as follows (forward/reverse): GATA4, gggccctttgtcattcttc / tccttgctttctgcctgctac; MEF2C, gatgaatgaagctggaagg/ cacagctcgatctecaaaatc. The PCR products were subject to electrophoresis on 1.5% agarose gels, scanned, and semi-quantitated using Image-Quant software (Kodak 1D V3.53; Kodak, New Haven).

HDAC Activity Analysis
Nuclear extracts of LV tissue were obtained with a Nuclear Extraction Kit (Bio Vision). Nuclear extract was then analyzed for HDAC activity with a HDAC Colorimetric Activity Assay Kit (Bio Vision, K331-100) according to manufacturer’s instructions. The plate was read by a spectrophotometer at 400 nm absorbance and values were determined as O.D./µg protein in samples.

Intracellular Ca\(^{2+}\) Levels by Ca\(^{2+}\) Fluorescent Dye Indo 1
Intracellular Ca\(^{2+}\) levels were measured with the Ca\(^{2+}\) fluorescent dye indo 1 (Dojin Kagaku). The ratio of 400-nm fluorescence to 500-nm fluorescence, which was collected from the cultured cardiomyocytes of neonatal rats illuminated by 360-nm light, was used as an indicator for intracellular Ca\(^{2+}\) concentration.

CaMKII Activities
The activity of CaMKII was assayed by measuring the phosphorylation of synthetic peptide autocamtide-2, a peptide substrate for CaMKII. In brief, total proteins isolated from cultured cardiomyocytes were immunoprecipitated using an anti-CaMKII polyclonal antibody (Santa Cruz Biotechnology Inc.) and the immunocomplex was incubated with autocamtide-2 and [γ-\(^{32}\)P]ATP at the presence of Ca\(^{2+}\) and CaM. After incubation, the autocamtide-2 was collected using Whatman P81 paper and counted according to the Cerenkov's method.

Calcineurin (CnA) Activities
The activity of CnA was determined with phosphorylated GST-RII peptide as a substrate. We separated CaM-bound CnA (active CnA > 100 kDa) from free CnA (inactive CnA < 100 kDa) using Ultrafree-MC centrifugal filter units (Millipore).

Statistical Analysis
All values are expressed as mean ± S.D. of more than three experiments. Comparison between two groups was analyzed by the two-tailed, unpaired Student’s t-test (normality) or non-parametric test (non-normality, Mann-Whitney U-test). We used Kolmogorov-Smirnov test for checking the normality. The normality was assumed by P value > 5. If P value < 5, the data weren’t normal distribution, we used non-parametric test for analysis. Multiple group comparison was made by one-way ANOVA followed by Dunnett’s modified t test for comparison of means. Values of P < 0.05 were considered statistically significant.
Supplement Results

Upregulation of RyR-2 Phosphorylation by PKA in RyR-2 Deficient Heart
At first, we confirmed that PKA phosphorylation completely dissociated FKBP12.6 from RyR-2 in WT mice (Figure 1, WT with PKA). The amount of FKBP12.6-associated RyR-2 was less in RyR-2<sup>−/−</sup> heart compared to WT heart, whereas PKA-phosphorylated RyR-2 in RyR-2<sup>+/−</sup> heart was more than WT one under basal conditions (Figure S1). TAC for 3 weeks decreased the amount of FKBP12.6-associated RyR-2 not only in WT mice but also in RyR-2<sup>−/−</sup> mice. TAC also increased the PKA-phosphorylated RyR-2 in both type of mice (Figure S1).

Affect of RyR-2 Deficiency on TAC-induced GATA2 and MEF2C Expression and HDACs Activity
There were no any significant differences in GATA2 and MEF2C expression and HDACs activity between Sham-operated WT and RyR-2<sup>+/−</sup> mice (Figure S2). After TAC, the increases in GATA4 mRNA expression in the heart of WT mice were significantly suppressed in RyR-2<sup>−/−</sup> mice, whereas the increase in MEF2C mRNA expression was not changed between the two types of mice. The increase in activity of HDAC in nuclear proteins in LV tissues after TAC for 3 weeks was not different between WT and RyR-2<sup>−/−</sup> mice. Our data suggest that CaMKII rather than calcineurin regulates activity of HDAC and transcription activation of MEF2C.

Downregulation of p70S6 Kinase Activity in the Heart of RyR-2<sup>+/−</sup> Mice after TAC
There was no significant difference in p70S6 kinase activity between Sham-operated WT and RyR-2<sup>−/−</sup> mice (Figure S3). The kinase activity was significantly elevated at 3 weeks after TAC in the heart of WT mice and the increase was suppressed in the heart of RyR-2<sup>−/−</sup> mice, consisting with the results of phosphorylation of Akt1.

Intracellular Ca<sup>2+</sup> Levels and Activities of CaMKII and CnA
We used angiotensin II (AngII) as a stimulator for the intracellular Ca<sup>2+</sup> levels and CaMKII and CnA activation since AngII is greatly involved in pressure overload-induced cardiac hypertrophy. AngII (10 nmol/L) could not induce increases in cytosolic free Ca<sup>2+</sup> levels at both the systolic and diastolic phases in cultured cardiomyocytes of neonatal rats (Figure S4A). Although AngII rapidly (at 1 min) increased activities of CaMKII in a dose-dependent manner, it could not induce an increase in CnA activation (Figure S4B). These results suggest that CaMKII, but not CnA, could be activated without a global increase in Ca<sup>2+</sup> levels in the cytoplasm.
References


Cardiac myocytes (CM) were isolated from adult male WT and \(RyR-2^{+/−}\) mice with (+) or without (-) TAC for 3 weeks and intracellular Ca\(^{2+}\) concentration and contraction were analyzed. \(dCa^{2+}/dt\) of systolic indicates the rate of an increase in intracellular Ca\(^{2+}\) concentration; T1/2 of diastolic phase indicates the half-time of relaxation from caffeine-induced contracture; FS% indicates percentile fractional shortening of myocyte length. Data are shown as mean ± S.D. of 20~28 cardiomyocytes from 3 WT and 3 \(RyR-2^{+/−}\) mice, respectively. * \(P < 0.01\), † \(P < 0.05\) vs WT mice without TAC; ‡ \(P < 0.01\), § \(P < 0.05\) vs WT mice with TAC. \(P\)-values indicate the comparison within the same genotype mice. NS, none statistically significant.

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT</th>
<th>RyR-2(^{+/−})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAC</td>
<td>(P)-value</td>
</tr>
<tr>
<td>Heart (n)</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>CM (n)</td>
<td>25</td>
<td>+</td>
</tr>
<tr>
<td>(Ca^{2+}) transient amplitude</td>
<td>1.15 ± 0.08</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>(dCa^{2+}/dt) of systolic phase</td>
<td>0.031 ± 0.004</td>
<td>0.024 ± 0.005</td>
</tr>
<tr>
<td>T1/2 of diastolic phase</td>
<td>349 ± 10</td>
<td>387 ± 17</td>
</tr>
<tr>
<td>FS%</td>
<td>5.0 ± 0.8</td>
<td>3.9 ± 0.7</td>
</tr>
</tbody>
</table>
**Table S2.** Effects of Ryanodine on contraction of adult cardiomyocytes.

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT</th>
<th>RyR-2(^{+/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ryanodine</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Heart (n)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>CM (n)</td>
<td>5.2 ± 0.7</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>FS %</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Cardiac myocytes (CM) were isolated from adult male WT and RyR-2\(^{+/-}\) mice. Percentile fractional shortening of myocyte length (FS\%) was measured at the absent (-) or presence (+) of 100 μmol/L Ryanodine during electrical stimulation. Data are shown as mean ± S.D. of 18 and 20 cardiomyocytes from 3 WT and 3 RyR-2\(^{+/-}\) mice, respectively. P-values indicate the comparison within the same genotype mice. NS, none statistically significant.
**Figure S1.** Association of FKBP12.6 and PKA with RyR-2. WT and *RyR-2<sup>−/−</sup>* mice were subjected to Sham or TAC for 3 weeks. Association of RyR-2 with FKBP12.6 and PKA was examined by western blot analyses for FKBP12.6 and PKA after immunoprecipitated with an anti RyR-2 antibody. Phosphorylation of RyR-2 from WT mice with PKA (WT and PKA) was performed as described in Methods. Representative blots from 3 independent experiments are shown.
Figure S2

**Figure S2.** Expression of GATA4 and MEF2C genes and activity of HDACs. WT and RyR-2<sup>+/−</sup> mice were subjected to Sham or TAC for 3 weeks. **A.** Expression of GATA4 and MEF2C genes analyzed by RT-PCR. Representative photographs form 5 independent experiments are shown. **B,** HDAC activities. Data are expressed as mean ± S.D. from 5 hearts. *P < 0.05 vs sham-operated mice of the same genotype.
**Figure S3.** Activity of p70S6 kinase. WT and RyR-2<sup>−/−</sup> mice were subjected to Sham or TAC for 2 hours (2h) and 3 weeks (3W). p70S6 kinase activity was measured as described in Methods. Data are expressed as mean ± S.D. from 5 hearts. * P < 0.05 vs sham-operated mice of the same genotype; † P < 0.05 vs respective WT-TAC group.
Figure S4

A

![Graph showing Indo-1 ratio (nmol/L) over time after AngII (10 nmol/L) stimulation.](image)

- **Indo-1 ratio (nmol/L)**
- **1 sec**
- **1 min**
- **AngII (10 nmol/L)**

B

![Bar graph showing relative activation fold of CaMKII and Calcineurin with AngII concentrations.](image)

- **Relative activation (fold)**
- **CaMKII**
- **Calcineurin**
- **AngII concentrations (μmol/L)**
- **0**
- **0.001**
- **0.01**
- **0.1**
- **1**
- **10**

*Significant differences indicated with asterisks.*
**Figure 4.** A, AngII-induced changes in intracellular Ca$^{2+}$ levels in cardiomyocytes. Intracellular Ca$^{2+}$ concentration was measured with fluorescent dye indo 1. Representative tracing of intracellular Ca$^{2+}$ concentration transients before and after addition of AngII (10 nmol/L) is shown. B, Activation of CaMKII and CnA by AngII in cardiomyocytes. Cardiomyocytes were incubated with AngII at indicated concentration for 5 min. CaMKII and CnA activities were determined as described in Methods. Data represent mean ± S.D. from 3 independent experiments. *$P < 0.05$ vs control.