

Accelerated Development of Pressure Overload–Induced Cardiac Hypertrophy and Dysfunction in an RyR2-R176Q Knockin Mouse Model

Ralph J. van Oort, Jonathan L. Respress, Na Li, Corey Reynolds, Angela C. De Almeida, Darlene G. Skapura, Leon J. De Windt, Xander H.T. Wehrens

Abstract—In response to chronic hypertension, the heart compensates by hypertrophic growth, which frequently progresses to heart failure. Although intracellular calcium (Ca^{2+}) has a central role in hypertrophic signaling pathways, the Ca^{2+} source for activating these pathways remains elusive. We hypothesized that pathological sarcoplasmic reticulum Ca^{2+} leak through defective cardiac intracellular Ca^{2+} release channels/ryanodine receptors (RyR2) accelerates heart failure development by stimulating Ca^{2+} -dependent hypertrophic signaling. Mice heterozygous for the gain-of-function mutation R176Q/+ in RyR2 and wild-type mice were subjected to transverse aortic constriction. Cardiac function was significantly lower, and cardiac dimensions were larger at 8 weeks after transverse aortic constriction in R176Q/+ compared with wild-type mice. R176Q/+ mice displayed an enhanced hypertrophic response compared with wild-type mice as assessed by heart weight:body weight ratios and cardiomyocyte cross-sectional areas after transverse aortic constriction. Quantitative PCR revealed increased transcriptional activation of cardiac stress genes in R176Q/+ mice after transverse aortic constriction. Moreover, pressure overload resulted in an increased sarcoplasmic reticulum Ca^{2+} leak, associated with higher expression levels of the exon 4 splice form of regulator of calcineurin 1, and a decrease in nuclear factor of activated T-cells phosphorylation in R176Q/+ mice compared with wild-type mice. Taken together, our results suggest that RyR2-dependent sarcoplasmic reticulum Ca^{2+} leak activates the prohypertrophic calcineurin/nuclear factor of activated T-cells pathway under conditions of pressure overload. (*Hypertension*. 2010;55:932-938.)

Key Words: calcium ■ heart failure ■ hypertrophy ■ ryanodine receptor calcium release channel ■ sarcoplasmic reticulum

Cardiac hypertrophy is an adaptive physiological mechanism that develops in response to hypertension-associated functional overload of the heart to preserve cardiac output and tissue perfusion.¹ Despite these initial benefits, cardiac hypertrophy is also an important independent risk factor for cardiovascular disease, and, if left untreated, it often deteriorates into decompensation and heart failure.² It is now well established that several major signaling pathways involved in the hypertrophic response are activated by increases in intracellular calcium (Ca^{2+}) concentrations.³

Sustained elevations in cytosolic Ca^{2+} levels activate the Ca^{2+} /calmodulin-dependent serine-threonine phosphatase calcineurin (CaN).⁴ Once activated, CaN dephosphorylates members of the nuclear factor of activated T-cells (NFAT) transcription factor family, resulting in translocation of NFAT into the nucleus and the induction of hypertrophic gene expression.² Another important Ca^{2+} -activated cardiac hypertrophy signaling pathway involves Ca^{2+} /calmodulin-dependent protein kinase (CaMKII)-mediated phosphorylation of class II histone deacetylases (HDACs).

Upon phosphorylation, HDACs are exported out of the nucleus, thereby derepressing activity of the myocyte enhancer factor 2 family of transcription factors.^{2,5}

Despite the central role of Ca^{2+} -dependent hypertrophic signaling cascades, the subcellular origin of Ca^{2+} signals that activate these signaling pathways in cardiac myocytes remains mostly unknown.^{2,5} The question of whether altered patterns of Ca^{2+} release and reuptake associated with excitation-contraction coupling may affect hypertrophic signaling pathways remains very controversial.⁶

In cardiomyocytes, excitation-contraction coupling initiated by the influx of extracellular Ca^{2+} through the L-type Ca^{2+} channel increases Ca^{2+} levels in the junctional space between transverse (T) tubules and the sarcoplasmic reticulum (SR).⁷ This increase in local Ca^{2+} levels activates ryanodine receptor (RyR2)/intracellular Ca^{2+} release channels, which release a much greater amount of Ca^{2+} from the SR known as the contractile Ca^{2+} transient.^{6,8} Myocyte relaxation occurs when cytosolic Ca^{2+} levels are subsequently reduced to baseline levels

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From the Department of Molecular Physiology and Biophysics (R.J.v.O., J.L.R., N.L., C.R., A.C.D.A., D.G.S., X.H.T.W.), and the Department of Medicine (Cardiology) (X.H.T.W.), Baylor College of Medicine, Houston, Tex; Department of Cardiology, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands (L.J.D.W.).

Correspondence to Xander H.T. Wehrens, Baylor College of Medicine, 1 Baylor Plaza, BCM 335, Houston, TX 77030. E-mail wehrens@bcm.edu
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through the actions of Ca^{2+} recycling proteins, such as SR Ca^{2+} -ATPase and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger.⁷ It has been proposed that enhanced Ca^{2+} leak from the SR during diastole, caused by “leaky” RyR2 Ca^{2+} release channels, may underlie contractile dysfunction.^{8–12} However, it is controversial at present whether this diastolic SR Ca^{2+} leak might also activate Ca^{2+} -dependent hypertrophic signaling pathways under pathological conditions.

Here, we tested the hypothesis that enhanced SR Ca^{2+} leak through RyR2 accelerates the development of cardiac hypertrophy through the activation of Ca^{2+} -dependent signaling pathways. To study the specific effects of increased RyR2-mediated Ca^{2+} release, we studied knockin mice heterozygous for mutation R176Q in RyR2 (R176Q/+). This mutation was shown previously to increase SR Ca^{2+} release in atrial myocytes and in ventricular myocytes after catecholaminergic stimulation.^{13,14}

Methods

An expanded Methods section is available in the online Data Supplement at <http://hyper.ahajournals.org>.

Transverse Aortic Constriction in Mice

R176Q/+ knockin mice were generated as described previously.¹³ Transverse aortic constriction (TAC) was performed as described previously in detail.¹⁵

Transthoracic Echocardiography

Cardiac function was assessed by echocardiography using a VisualSonics VeVo 770 Imaging System (VisualSonics), as described previously.¹⁶

Invasive Hemodynamic Measurements

Pressure-volume relationships were generated by cardiac catheterization of a 1.4F catheter (Millar Instruments) and analyzed using the IOX data acquisition system (Emka Technologies).

Histology

Paraffin sections of the hearts were stained with hematoxylin-eosin for cell morphology. Myocyte cross-sectional areas were measured in sections stained with fluorescein-conjugated wheat germ agglutinin.

Quantitative RT-PCR

Real-time PCR was performed using a Mastercycler ep realplex (Eppendorf), as described previously.¹⁷

Calcium Imaging in Isolated Ventricular Myocytes

Single ventricular myocyte isolation and Ca^{2+} imaging were performed as described in detail previously.^{14,16}

Immunoprecipitation and Western Blot Analysis

CaMKII autophosphorylation status was determined by Western blot on mouse heart homogenates using anti-CaMKII δ (1:1000) and antiThr287-CaMKII (1:1000; Cayman Chemicals) antibodies. To determine NFAT phosphorylation status, 500 μg of protein extract were immunoprecipitated with anti-NFATc3 (Santa Cruz Biotechnology) and blotted with anti-phosphoserine (Invitrogen).

Statistical Analysis

Results are expressed as mean \pm SEM. A value of $P<0.05$ was considered statistically significant.

Results

Accelerated Cardiac Failure in R176Q/+ Knockin Mice

R176Q/+ knockin mice ($n=28$) and wild-type (WT) littermates ($n=32$) with an average weight of 24.2 ± 1.1 g and 23.1 ± 1.0 g, respectively, were used for this study. At baseline, left ventricular (LV) contractility and dimensions measured by echocardiography were similar in both groups (see Table S1 in the online Data Supplement), consistent with previous studies showing the absence of cardiac hypertrophy in R176Q/+ mice.¹³

After baseline echocardiography measurements, pressure overload was induced surgically by TAC in R176Q/+ ($n=19$) and WT ($n=22$) mice. In addition, 9 R176Q/+ and 10 WT mice were subjected to a sham procedure. The presence of a pressure gradient was evaluated using Doppler ultrasound measurements 7 days after surgery and was similar in R176Q/+ TAC and WT TAC mice. To evaluate the effects of the R176Q/+ mutation in RyR2 on the development of cardiac failure after pressure overload, LV function was evaluated using serial echocardiography 2, 4, and 8 weeks after TAC. Measurements were made at the same level of anesthesia in all of the mice, and body temperature was carefully maintained between 36°C and 37°C . There were no significant differences in heart rates comparing the experimental groups of mice (heart rate at 8 weeks after TAC: WT, 496 ± 10 bpm; R176Q/+, 500 ± 8 bpm; P value not significant). R176Q/+ TAC mice exhibited an accelerated development of contractile failure compared with WT TAC mice (Figure 1). At each time point, ejection fractions were significantly lower in R176Q/+ TAC mice compared with WT TAC mice (Figure 1A). Moreover, R176Q/+ mice developed more pronounced cardiac dilatation after pressure overload (Figure 1B). The increase in end-diastolic LV diameters 8 weeks after TAC was significantly larger in R176Q/+ mice ($32.5\pm 3.0\%$) compared with WT mice ($26.5\pm 2.9\%$; $P<0.05$; Figure 1B). There were no significant changes in ejection fraction or end-diastolic LV diameters in either R176Q/+ or WT mice after the sham procedure (data not shown).

At the conclusion of the experiment 8 weeks after TAC, pressure-volume loops were obtained using LV catheterization. These hemodynamic measurements revealed a significantly lower first derivative of LV pressure over time (dP/dt_{max}) in R176Q/+ TAC mice ($n=12$) compared with WT TAC mice ($n=14$; $P<0.05$), suggesting that the R176Q mutation in RyR2 accelerates the depression in systolic function in R176Q/+ mice after pressure overload (Figure 1C). Furthermore, quantification of the peak negative dP/dt index revealed a strong trend toward more severely impaired diastolic function in R176Q/+ TAC mice (-4268.9 ± 330.7 mm Hg/s) compared with WT TAC mice (-5033.2 ± 374.9 mm Hg/s; $P=0.07$) after pressure overload, although this did not reach statistical significance (Figure 1D).

R176Q Mutation Augments Cardiac Hypertrophy After Pressure Overload

Eight weeks after surgery, heart and cardiomyocyte sizes were indistinguishable between sham-operated R176Q/+ and WT mice (Figure 2A). The hypertrophic response observed after aortic constriction, however, was exacerbated in

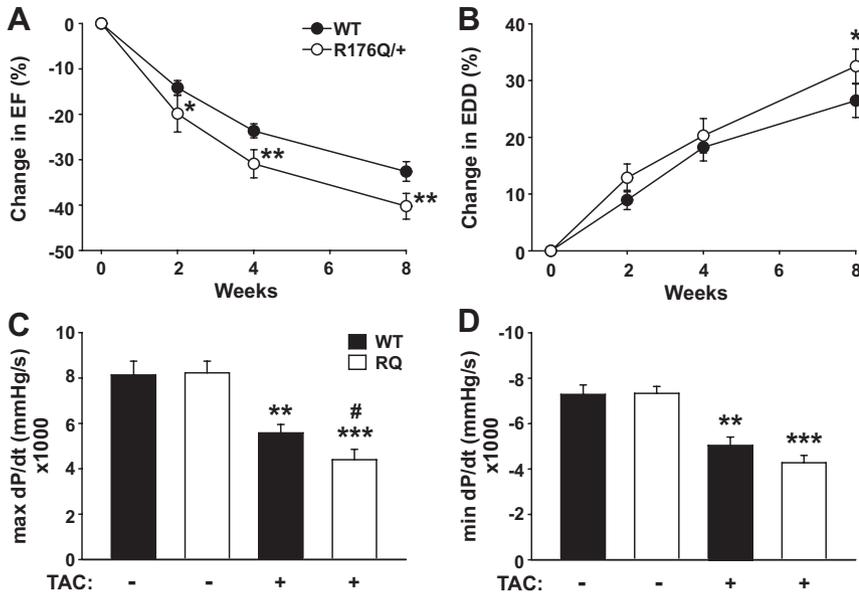


Figure 1. Accelerated cardiac contractile dysfunction in R176Q/+ mice after pressure overload. A and B, Relative change in ejection fraction (EF) (A) and end-diastolic dimension (EDD) (B) 2, 4, and 8 weeks after TAC in R176Q/+ (n=15) and WT (n=17) mice, normalized to pre-TAC baseline values. * $P<0.05$, ** $P<0.01$ vs WT. C and D, Pressure-volume loop measurements demonstrate loss of cardiac contractility in R176Q/+ TAC mice (n=12) compared with WT TAC (n=14) 8 weeks after TAC. ** $P<0.01$, *** $P<0.001$ vs corresponding sham, # $P<0.05$ vs WT TAC. Data represent average \pm SEM.

R176Q/+ mice compared with WT mice. Indeed, heart weight (HW):body weight ratio was larger in R176Q/+ mice (13.3 ± 1.2 mg/g) compared with WT mice (10.3 ± 0.7 mg/g) after induction of pressure overload ($P<0.05$; Figure 2B). Consistently, HW:tibia length ratio was also larger in R176Q/+ mice (16.2 ± 1.0 mg/mm) compared with WT mice (13.2 ± 0.7 mg/mm) 8 weeks after TAC ($P<0.05$; Figure 2C). In contrast, HW:body weight and HW:tibia length ratios were similar in sham-operated R176Q/+ and WT mice.

To further test whether the R176Q mutation in RyR2 affected the hypertrophic response after pressure overload, we measured LV wall thickness using echocardiography. Figure 2D shows that R176Q/+ mice exhibited a more pronounced hypertrophic response after TAC, as evidenced by a significantly larger LV wall thickness (1.1 ± 0.02 mm) compared with WT TAC mice (1.0 ± 0.01 mm; $P<0.01$).

As a quantitative evaluation of individual myofiber hypertrophy, we measured individual myofibril cross-sectional areas from wheat germ agglutinin-stained sections (Figure 2A and 2E). Sham-operated R176Q/+ and WT mice had similar myofiber cross-sectional areas, whereas R176Q/+ mice subjected to TAC exhibited a significantly increased myofiber size ($420.8 \pm 8.3 \mu\text{m}^2$) compared with WT TAC mice ($352.6 \pm 6.5 \mu\text{m}^2$; $P<0.001$; Figure 2E). Enlargement of myofiber diameters occurred throughout the ventricular wall and was not limited to specific areas or layers within the wall.

A hallmark of the stress-associated cardiac remodeling process induced by pressure overload is reactivation of fetal cardiac genes. Therefore, the expression levels of a number of fetal genes were determined using quantitative PCR (Figure 2F and 2G). Transcripts levels for *acta1* (α -skeletal muscle actin; Figure 2F), and *nppb* (brain natriuretic peptide; Figure 2G), were higher in R176Q/+ mice compared with WT mice 8 weeks after TAC. In sham-operated animals, there were no differences between any of these transcript levels comparing WT and R176Q/+ mice. Overall, these data suggest that the gain-of-function mutation R176Q in RyR2 augments the myofiber hypertrophy response after biomechanical stress.

Elevated SR Ca^{2+} Leak and Activation of NFAT-Dependent Hypertrophic Signaling in Cardiomyocytes From R176Q/+ Mice

To determine whether pressure overload resulted in an increased SR Ca^{2+} leakiness in R176Q/+ mice, isolated cardiomyocytes were loaded with a Ca^{2+} -sensitive dye, and SR Ca^{2+} leak was measured using the protocol described by Shannon et al¹⁸ (Figure 3A). There was a nonsignificant trend toward an increased Ca^{2+} leak in WT TAC (7.6 ± 0.7 arbitrary units [AU]) compared with WT sham animals (5.8 ± 0.7 AU; $P=0.07$). The magnitude of SR Ca^{2+} leak was significantly larger in R176Q/+ TAC mice (10.2 ± 1.0 AU) compared with R176Q/+ sham (6.8 ± 0.4 AU; $P<0.01$) and WT TAC (7.6 ± 0.7 AU; $P<0.05$) animals (Figure 3B).

Because an increased level of cytosolic Ca^{2+} might activate Ca^{2+} -dependent hypertrophic signaling pathways, we determined whether there is an increased activation of either the CaMKII/HDAC or CaN/NFAT pathway in R176Q/+ TAC mice (Figure 4). Activation of the CaMKII/HDAC hypertrophic signaling pathway was assessed by Western blotting using antibodies against autophosphorylated CaMKII (Figure 4A). There was a trend toward increased levels of CaMKII autophosphorylation of WT and R176Q/+ mice after TAC (WT TAC versus sham: $P=0.17$; R176Q/+ TAC versus sham: $P=0.10$). However, there was no significant difference in CaMKII autophosphorylation comparing R176Q/+ and WT mice 8 weeks after TAC (Figure 4B), suggesting similar CaMKII activity levels in these mice. No differences in total CaMKII levels were detected between groups when normalized for GAPDH protein level (data not shown).

To probe activity of the CaN/NFAT pathway, we determined the phosphorylation level of NFAT (Figure 4C). NFAT was found to be dephosphorylated in R176Q/+ mice at 8 weeks after TAC (Figure 4D), indicating an enhanced activity of the phosphatase CaN in these mice. A Western blot for GAPDH on 10% of the input samples was used to verify equal protein levels in each group (data not shown). Next, we assessed transcript levels of the NFAT-regulated exon 4 splice isoform of *rcan1* (regulator

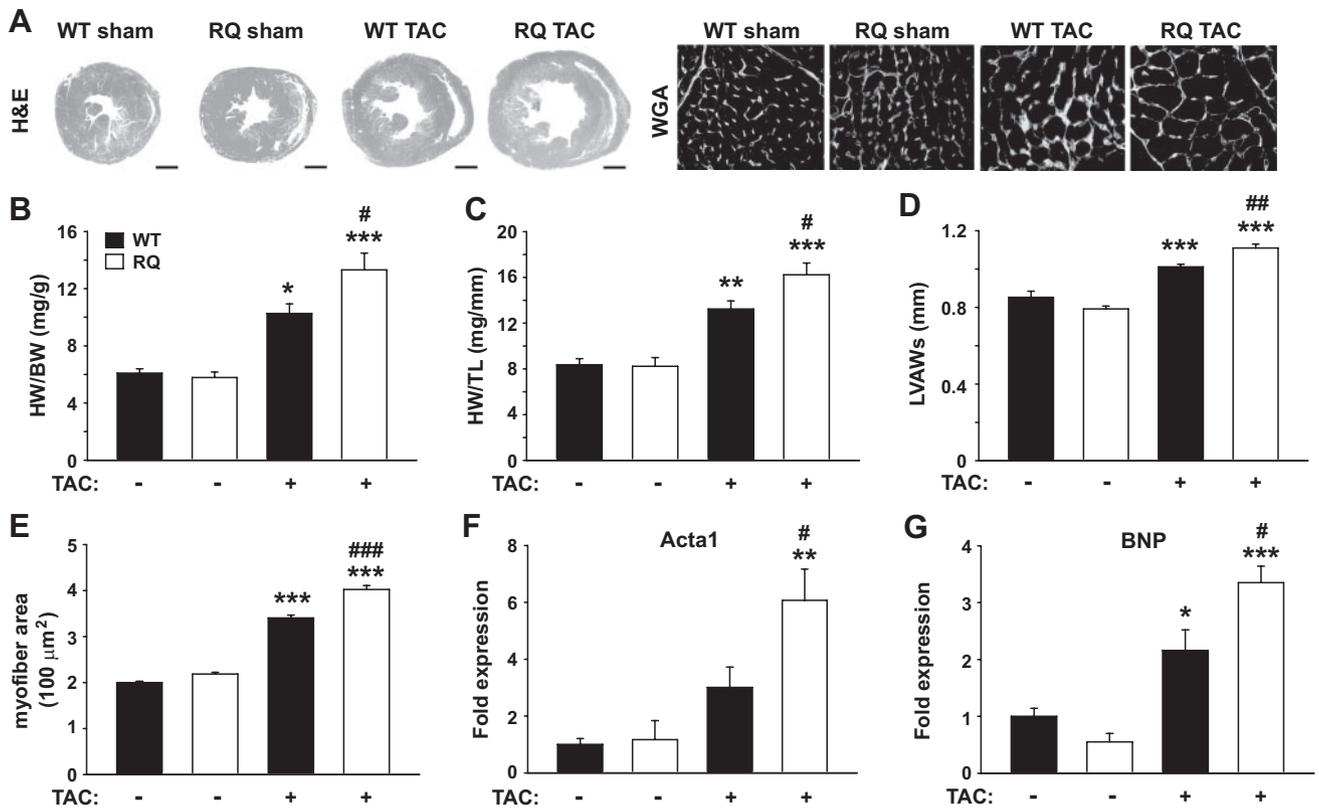


Figure 2. Augmented cardiac enlargement and myocyte hypertrophy in R176Q/+ mice after TAC. A, Hematoxylin-eosin (H&E)-stained sections cut at the midsagittal level of the heart and representative photomicrographs illustrating ventricular myocyte cross-sections stained with wheat germ agglutinin (WGA). Scale bar: 1 mm. B, HW:body weight (HW/BW) ratios 8 weeks after TAC. C, HW:tibia length (HW/TL) ratios 8 weeks after TAC. D, LV anterior wall thickness during systole, as determined by echocardiography 8 weeks after TAC. E, Quantification of cardiomyocyte cross-sectional area measurements in sham-operated WT (269 cells from 3 hearts) and R176Q/+ (257 cells from 4 hearts) mice and 8 weeks post-TAC in (WT 259 cells from 3 hearts) and R176Q/+ (319 cells from 3 hearts) mice. F and G, Verification of mRNA levels of cardiac stress genes α -skeletal muscle actin (Acta1) (F) and brain natriuretic peptide (BNP) (G) by real-time PCR analysis. Transcript levels in WT sham group were set at 1. $n \geq 6$ per group. Data represent average \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs corresponding sham group. # $P < 0.05$, ### $P < 0.01$, ### $P < 0.001$ R176Q/+ TAC vs WT TAC.

of CaN-1; Figure 4E).¹⁹ Pressure overload induced an increase in the RCAN1-4 expression level in WT mice (3.1 ± 1.4 for WT TAC versus 1.0 ± 0.3 for WT sham; $P < 0.05$). RCAN1-4 expression level was more upregulated in R176Q/+ mice (RQ; RQ TAC: 7.3 ± 1.0) than in WT mice (WT TAC = 3.1 ± 1.4) after 8 weeks of pressure overload (RQ TAC versus WT TAC:

$P < 0.05$), reflecting an increase in total NFAT activity downstream of CaN signaling in the heart.²⁰ Thus, these results indicate that the R176Q gain-of-function mutation in RyR2 led to an augmentation of CaN-induced structural alterations in the myocardium. Together, these data suggest that the accelerated development of heart failure in R176Q/+ mice might be

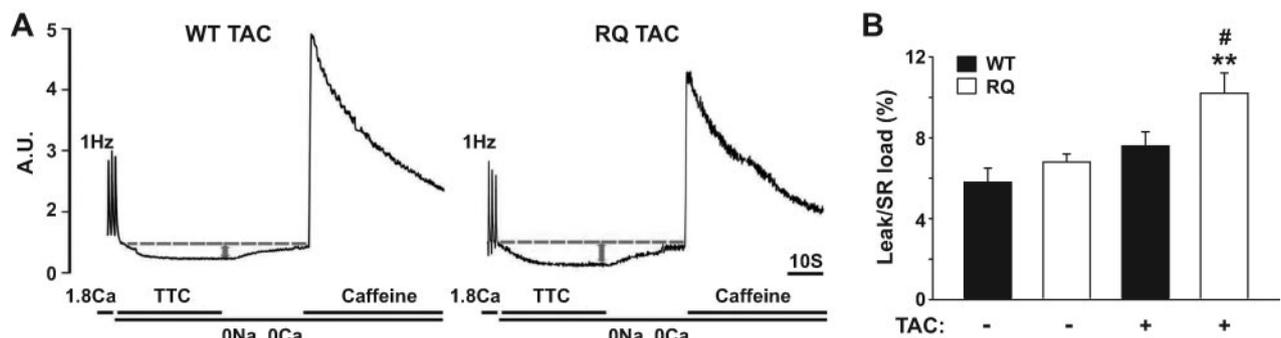


Figure 3. Increased SR Ca^{2+} leak in cardiomyocytes from R176Q/+ mice after TAC. A, Representative $[\text{Ca}^{2+}]_i$ recordings obtained from fluo-4 AM-loaded cardiomyocytes from WT TAC and R176Q/+ TAC mice during 1-Hz pacing in 1.8-mmol/L Ca^{2+} , followed by a rapid switch to 0 Na^+ , 0 Ca^{2+} tyrode solution and tetracaine (TTC). SR Ca^{2+} content is measured by changing superfusate to 10 mmol/L of caffeine in 0 Na^+ , 0 Ca^{2+} tyrode. SR Ca^{2+} leak was measured as indicated by the arrows. B, Quantification of SR Ca^{2+} leak normalized to SR Ca^{2+} content. Data were obtained from 20 to 33 cells obtained from 3 mice per group. ** $P < 0.01$ vs corresponding sham; # $P < 0.05$ vs WT TAC.

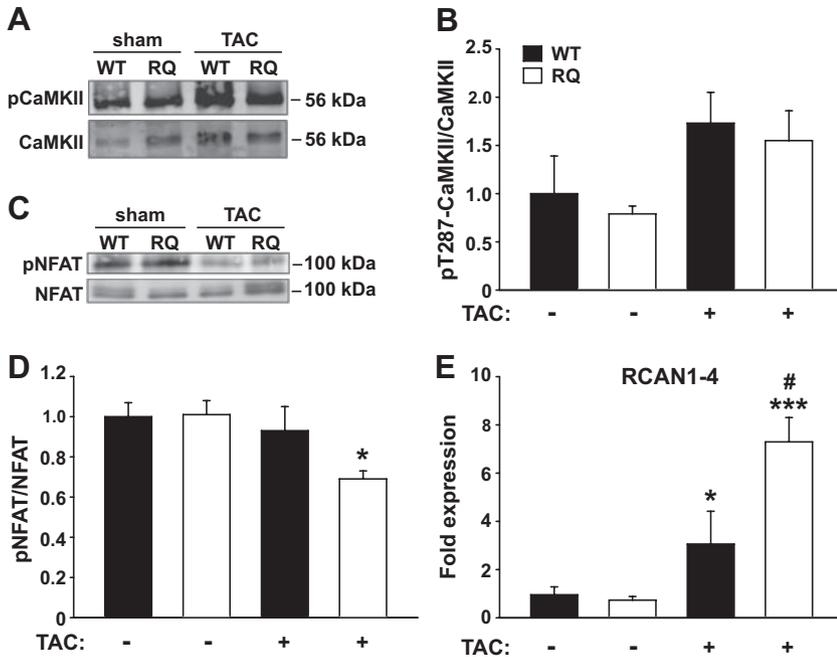


Figure 4. Increased CaN/NFAT activity in R176Q/+ mice after TAC. A, Representative Western blots for phosphorylated CaMKII and total CaMKII in lysates from WT and RQ mice after sham or TAC surgery. B, Quantification of CaMKII phosphorylation indicates equal CaMKII activity WT and RQ TAC hearts. n≥6 per group. C, Western blot analysis of phosphorylated and total NFAT in lysates from WT and RQ mice after sham or TAC surgery. D, Quantification of NFAT phosphorylation reveals an increase in CaN activity in RQ TAC hearts. n=4 per group. E, Verification of mRNA levels of CaN-NFAT target gene RCAN1-4 by real-time PCR analysis. Transcript levels in WT sham group were set at 1. n≥10 per group. *P<0.05, ***P<0.001 vs corresponding sham group, #P<0.05 vs WT TAC.

predominantly mediated by excessive activation of the Ca²⁺/CaN/NFAT signaling pathway as a result of enhanced SR Ca²⁺ leak via RyR2.

Discussion

It has been well recognized that Ca²⁺-dependent signaling pathways play a key role in the development of pathological cardiac hypertrophy.² Although these intracellular signaling events have been well described during the past decade, the exact nature of the subcellular Ca²⁺ pool that initiates hypertrophic signaling has remained quite controversial.⁶ The source of this “hypertrophy-associated” Ca²⁺ pool might be an influx of extracellular Ca²⁺ via a voltage-gated Ca²⁺ channel²¹⁻²³ or store-operated Ca²⁺ channels,²⁴⁻²⁶ release from the SR via RyR2,¹² or release from the nucleus via inositol triphosphate

receptors.²⁷ Our results suggest that pathological leak of Ca²⁺ from the SR through mutant RyR2 accelerates the development of cardiac hypertrophy and heart failure after pressure overload. These data are also consistent with previous reports suggesting that increased diastolic RyR2 Ca²⁺ leak impairs cardiac contractility because of a secondary decrease in SR Ca²⁺ loading.²⁸⁻³⁰ In addition, our results provide the first in vivo evidence that increased RyR2-mediated Ca²⁺ release from the SR leads to activation of the Ca²⁺-dependent CaN/NFAT hypertrophic signaling pathway.

Defects in RyR2 Ca²⁺ channel regulation are believed to play a central role in the development of contractile dysfunction in heart failure.³¹ A variety of alterations in the subunit composition of the RyR2 macromolecular complex have been demonstrated in patients with congestive heart failure, including de-

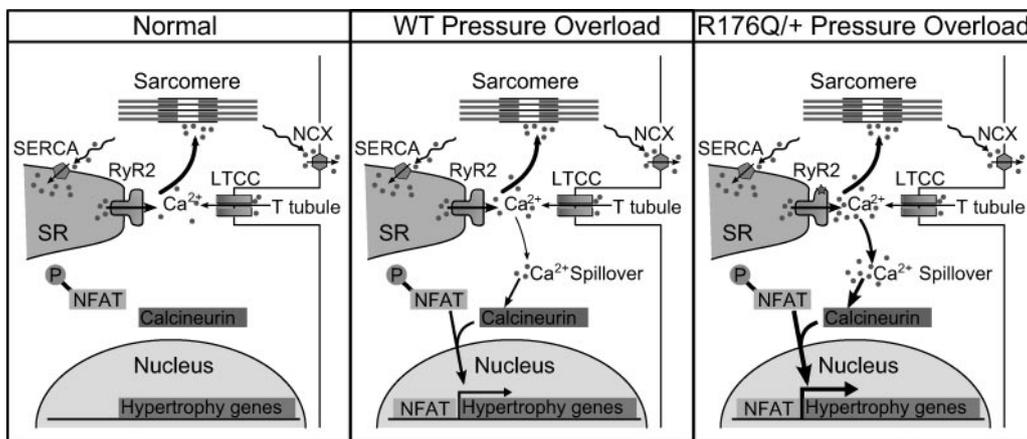


Figure 5. Calcium spillover from the calcium release unit triggers hypertrophic signaling. In the normal heart, Ca²⁺ released from the SR is used for contraction. During diastole, Ca²⁺ is removed from the cytosol by reuptake via SR Ca²⁺-ATPase (SERCA2a) and cellular excretion via the Na⁺/Ca²⁺-exchanger (NCX1). A pathological increase in RyR2 open probability during diastole because of pressure overload causes a greater release of Ca²⁺ from the SR, resulting in spillover of Ca²⁺ from the cleft between SR and T tubule. This spillover, which is greater in mice with R176Q/+ mutation in RyR2, leads to an increase in CaN/NFAT signaling and an accelerated development of cardiac hypertrophy and heart failure.

creased levels of FK506-binding protein 12.6 (or calstabin2), protein phosphatase 1, protein phosphatase 2A, and phosphodiesterase 4D3.^{32–34} In addition, changes in RyR2 posttranslational modifications, such as oxidation, S nitrosylation, and phosphorylation, have been shown in patients and animal models with heart failure.^{35–40} The combination of these alterations of RyR2 leads to a decreased ability of the channel to remain closed during diastole, resulting in diastolic Ca²⁺ leak from the SR.^{41,42} Although it has been well accepted that increased diastolic SR Ca²⁺ releases contribute to reduced cardiac contractility in patients with decompensated hypertrophy or heart failure, significant controversy exists about the source of Ca²⁺ involved in the activation of Ca²⁺-dependent prohypertrophic signaling cascades.⁶

Our data suggest that pathological Ca²⁺ leak from the SR through leaky RyR2 may lead to enhanced activation of the CaN/NFAT-dependent hypertrophic signaling pathway (Figure 5). Our data are consistent with those obtained in mice in which the calmodulin binding site on RyR2 has been disrupted with 3 mutations (RyR2-W3587A/L3591D/F3603A). Homozygous RyR2-ADA knockin mice exhibit spontaneous SR Ca²⁺ release episodes, neonatal cardiac hypertrophy, and activation of HDAC4/myocyte enhancer factor 2 signaling.¹² Moreover, MCIP1 (RCAN1-4) levels were elevated, suggesting that the CaN pathway was activated in RyR2-ADA knockin mice.¹² In contrast, heterozygous RyR2-ADA mice did not exhibit signs of cardiac hypertrophy or transcriptional activation of genes associated with hypertrophy. Together, these data suggest that a minor defect in RyR2 (RyR2-ADA heterozygous and RyR2-R176Q/+ heterozygous mice) might not be sufficient to induce cardiac hypertrophy, whereas a more pronounced defect (RyR2-ADA homozygous) or a minor defect plus an exogenous stressor (chronic hypertension) may induce activation of hypertrophic signaling pathways.

Additional evidence has emerged from recent clinical studies showing that genetic defects in the *RyR2* gene may lead to a predisposition toward the development of hypertrophic cardiomyopathy and cardiac failure in patients.^{43–45} Two common single nucleotide polymorphisms in the human *RyR2* gene are associated with arrhythmogenic right ventricular cardiomyopathy, often associated with mild cardiac failure.⁴⁵ The presence of both single nucleotide polymorphisms in *RyR2* results in channels with a higher open probability, suggesting that the RyR2 leads to aberrant SR Ca²⁺ release under diastolic conditions. In addition, Fujino et al⁴⁶ identified missense mutation Thr1107Met and Gly2367Arg⁴⁷ patients with LV and asymmetrical septal hypertrophy.

Although it might seem counterintuitive that Ca²⁺-sensitive signaling pathways could be regulated by large contractile Ca²⁺ transients, recent studies suggest otherwise. Colella et al³ demonstrated in cultured neonatal cardiomyocytes that an increased frequency of Ca²⁺ transients, if prolonged for hours, is sufficient to activate the CaN/NFAT signaling pathway. These results are similar to those showing that an increased beating frequency of cultured neonatal myocytes or atrial tissue preparations induces NFAT translocation into the nucleus.^{48,49} Thus, changes in excitation-contraction coupling-associated Ca²⁺ fluxes might indeed mediate CaN activation and NFAT translocation, al-

though the mechanisms underlying this phenomenon are still unknown.

Perspectives

Our findings demonstrate that defective Ca²⁺ release from the SR via mutant RyR2 adversely affects cardiac remodeling, as seen during a sustained increase in blood pressure. Our study indicates that Ca²⁺ leaking from the SR enhances hypertrophic signaling, preferentially by activating the CaN/NFAT signaling pathway. This reveals a new role for SR Ca²⁺ in heart disease, but additional studies are required to test whether modification of its release might improve clinical outcome in cases of human cardiac disease.

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Disclosures

None.

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ONLINE SUPPLEMENT

Accelerated Development of Pressure Overload-Induced Cardiac Hypertrophy in a RyR2-R176Q Knockin Mouse Model

Ralph J. van Oort ^a, Jonathan L. Respress ^a, Na Li ^a, Corey Reynolds ^a, Angela C. De Almeida ^a, Darlene G. Skapura ^a, Leon J. De Windt ^b, Xander H.T. Wehrens ^{a,b,1}

^a From the Dept. of Molecular Physiology and Biophysics, and Medicine (Cardiology), Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, U.S.A.; ^b Department of Medical Physiology, University Medical Center Utrecht, Yalelaan 50, 3584 CM Utrecht, The Netherlands.

¹ Address correspondence to:
Xander H.T. Wehrens, MD PhD
Associate Professor
Baylor College of Medicine
One Baylor Plaza, BCM 335
Houston, TX 77030, U.S.A.
Tel: 713-798-4261
Fax: 713-798-3475
Email: wehrens@bcm.edu

EXPANDED ONLINE METHODS

Transverse aortic constriction (TAC) in mice

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, and were conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). TAC was performed as previously described in detail.¹ Wildtype (WT) and R176Q/+ knock-in mice of both genders were anesthetized using a mixture of 2% isoflurane and 95% O₂. The aortic arch was visualized by left anterior thoracotomy. TAC was created by subjecting the aorta to a defined, 27-gauge constriction using a 6-0 suture between the first and second truncus of the aortic arch. The sham procedure was identical except the aorta was not ligated. A computer-based Doppler signal processor (Indus Instruments, Houston, TX) was used to measure Doppler velocities in the right and left carotid arteries.² Right/left carotid peak velocity ratios were similar in WT TAC and R176Q/+ TAC groups. Mice were allowed to recover and were sacrificed 8 weeks after surgery.

Transthoracic echocardiography

For echocardiography, chest hair was removed and mice were anesthetized using 1.5% isoflurane in 95% O₂. Body temperature was maintained at 36-37°C on a heated platform, and electrocardiograms and temperature were continuously monitored. Cardiac function was assessed prospectively at two, four, and eight weeks following TAC using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described.³ Data analysis was performed using VisualSonics software (VisualSonics, Toronto, Canada). Volume in systole and diastole (V_s , V_d) were used to measure ejection fraction (EF) according to the following formula: $EF = [(V_d - V_s) V_d^{-1}] \times 100\%$.

Invasive hemodynamic measurements

Cardiac catheterization was used to assess left ventricular (LV) contractility using a 1.4F high-fidelity micromanometer catheter (Millar Instruments, Houston, TX). The catheter was advanced into the heart via the right carotid artery.⁴ Data were digitized and analyzed using the IOX data acquisition system (Emka Technologies, Falls Church, VA). The first derivative of LV pressure over time is expressed as dP/dt_{max} .

Histology

Hearts were cut in three sections transversally. The base and apex of the heart were flash-frozen in liquid nitrogen, whereas the middle transverse section was fixed in 10% buffered formalin for 48 hours. After paraffin embedding and sectioning, 5 μ m sections were stained with hematoxylin-eosin (H&E) for cell morphology. Myocyte cross-sectional areas were measured in sections stained with fluorescein-conjugated wheat germ agglutinin (WGA-FITC).⁵ Before staining, sections were deparaffinized in histoclear and hydrated by passage through a descending ethanol series to distilled water. To label the sarcolemma with WGA-FITC, sections were incubated for 30 min in 50 μ g/ml WGA-FITC (Sigma, Saint Louis, MO) in PBS, and rinsed in three changes of fresh PBS. The slides were mounted in vectashield with DAPI (Vector Laboratories,

Burlingame, CA). Sections were examined on an Olympus BH-2 epifluorescence microscope. At least 100 myocytes cut transversely were measured per animal, and 3-4 animals per group were studied in this experiment.⁶

Quantitative RT-PCR

One μg of RNA isolated from flash-frozen apex was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR using a Mastercycler ep realplex (Eppendorf, Hamburg, Germany), and fluorescence detection was performed in 96-well plates using SYBR Green.⁷ PCR amplification was performed as a singleplex reaction in a total reaction volume of 25 μl , consisting of 400 nM forward and reverse primers, 1 μl cDNA, 12.5 μl 2x SYBR Green PCR master mix (Invitrogen, Carlsbad, CA). The PCR was cycled between 95 °C/30 s and 60 °C/30 s for 40 cycles, following an initial denaturation step at 95 °C for 2 min. Primers were specific for mouse sequences and designed as described in detail before.⁷ Transcript quantities were compared using the relative Ct method, where the amount of target normalized to the amount of endogenous control (L7) and relative to the control sample is given by $2^{-\Delta\Delta\text{Ct}}$.

Calcium imaging in isolated ventricular myocytes

Single ventricular myocytes were isolated by a modified collagenase method as described previously.^{3, 8} Hearts were quickly removed and mounted on a Langendorff's apparatus and perfused with 0 Ca^{2+} normal Tyrode solution (NT; 137 NaCl mmol/L, 5.4 KCl mmol/L, 1 MgCl_2 mmol/L, 10 mmol/L glucose, 10 mmol/L HEPES, pH 7.4) at 3 mL/min for 5 min. Then the perfusate was switched to a collagenase II (0.8 mg/mL; Worthington, NJ) based enzymatic solution, and the heart was perfused for 8-10 min. After digestion, single ventricular myocytes were isolated from dissected and triturated ventricles and stabilized in 0 Ca^{2+} NT. Myocytes were kept in 500 μM Ca^{2+} NT at room temperature before use. For Ca^{2+} imaging, ventricular myocytes were loaded with 5 $\mu\text{mol/L}$ Fluo-4 AM (Invitrogen, Carlsbad, CA) for 30 min at room temperature (RT), followed by perfusion with 1.8mM Ca^{2+} NT to wash out extra fluorescent. Intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were measured using an illumination device (model Lambda DG-4, Sutter Instruments, Novato, CA), and an electro-multiplier intensified back-illuminated charge coupled device (CCD) camera (model Cascade 512B, Photometrics, Tucson, AZ). SR Ca^{2+} leak was measured as described in detail by Shannon et al.⁹ Myocytes were paced at 40V, 1Hz for 20 seconds, then quickly perfused with 5 $\mu\text{mol/L}$ tetracaine in 0 Na^+ 0 Ca^{2+} NT, followed by application of caffeine (10 mM) in 0 Na^+ , 0 Ca^{2+} NT to estimate steady-state SR Ca^{2+} . The tetracaine-dependent shift of Ca^{2+} from cytosol to SR (decrease in cytosolic $[\text{Ca}^{2+}]_i$ and increase in SR Ca^{2+} content) is proportional to SR Ca^{2+} leak in the absence of tetracaine.

Immunoprecipitation and Western blot analysis

Homogenates were prepared from the flash-frozen base of the hearts as described previously.³ For quantification of CaMKII autophosphorylation status, mouse heart homogenates were subjected to electrophoresis on 12% acrylamide gels, and transferred onto polyvinyl difluoride membranes. Membranes were probed with anti-CaMKII δ (1:1000) and anti-pThr287-CaMKII (1:1000; Cayman Chemicals, Ann Arbor, MI) antibodies at room temperature (RT) for 4 hours. To determine NFAT

phosphorylation status, 500 μ g of protein extract was immunoprecipitated with anti-NFATc3 (Santa Cruz Biotechnology, Santa Cruz, CA) and blotted with anti-phosphoserine (Invitrogen, Carlsbad, CA), as described previously.¹⁰ Membranes were then incubated with secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa-Fluor 680 (Invitrogen Molecular Probes, Carlsbad, CA) and IR800Dye (Rockland Immunochemicals, Gilbertsville, PA), respectively, and bands were quantified using infrared visualization and densitometry (Odyssey System, Lincoln, NE).

Statistical analysis

Results are expressed as mean \pm SEM. Statistical significance of differences between experimental groups was determined using Student paired t-test or ANOVA followed by Tukey's post-test when appropriate. A value of $P < 0.05$ was considered statistically significant.

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Table S1**Baseline left ventricular echocardiographic parameters in R176Q/+ and WT mice**

Parameter	WT (n=20)	R176Q/+ (n=19)	P value
Body weight (g)	24.2 ± 1.1	23.1 ± 1.0	N.S.
Heart rate (b.p.m.)	498 ± 13	504 ± 12	N.S.
Ejection Fraction (%)	68.0 ± 0.7	67.8 ± 1.3	N.S.
Fractional Shortening (%)	36.4 ± 0.5	37.4 ± 0.7	N.S.
End-systolic diameter (mm)	2.02 ± 0.03	2.06 ± 0.04	N.S.
End-diastolic diameter (mm)	3.14 ± 0.06	3.17 ± 0.05	N.S.
Anterior wall thickness (mm)	0.82 ± 0.01	0.81 ± 0.02	N.S.
Posterior wall thickness (mm)	0.89 ± 0.01	0.86 ± 0.01	N.S.