Role of RyR2 Phosphorylation at S2814 During Heart Failure Progression

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Rationale: Increased activity of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is thought to promote heart failure (HF) progression. However, the importance of CaMKII phosphorylation of ryanodine receptors (RyR2) in HF development and associated diastolic sarcoplasmic reticulum Ca\(^{2+}\) leak is unclear.

Objective: Determine the role of CaMKII phosphorylation of RyR2 in patients and mice with nonischemic and ischemic forms of HF.

Methods and Results: Phosphorylation of the primary CaMKII site S2814 on RyR2 was increased in patients with nonischemic, but not with ischemic, HF. Knock-in mice with an inactivated S2814 phosphorylation site were relatively protected from HF development after transverse aortic constriction compared with wild-type littermates. After transverse aortic constriction, S2814A mice did not exhibit pulmonary congestion and had reduced levels of atrial natriuretic factor. Cardiomyocytes from S2814A mice exhibited significantly lower sarcoplasmic reticulum Ca\(^{2+}\) leak and improved sarcoplasmic reticulum Ca\(^{2+}\) loading compared with wild-type mice after transverse aortic constriction. Interestingly, these protective effects on cardiac contractility were not observed in S2814A mice after experimental myocardial infarction.

Conclusions: Our results suggest that increased CaMKII phosphorylation of RyR2 plays a role in the development of pathological sarcoplasmic reticulum Ca\(^{2+}\) leak and HF development in nonischemic forms of HF such as transverse aortic constriction in mice. (Circ Res. 2012;110:1474-1483.)

Key Words: calcium ■ heart failure ■ ryanodine receptor ■ sarcoplasmic reticulum

Heart failure (HF) is a leading cause of morbidity and mortality and is responsible for one of every nine deaths in the United States alone. Recent studies have revealed that increased activity of the enzyme Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) plays a major role in the development of HF. CaMKII can promote pathological cardiac remodeling by increasing cell death, stimulating cardiac dilatation, promoting cardiac arrhythmias, and interfering with excitation–contraction coupling.2

Editorial, see p 1398
In This Issue, see p 1391

Excitation–contraction coupling is initiated by the influx of extracellular Ca\(^{2+}\) via voltage-gated Ca\(^{2+}\) channels, which triggers a much greater release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR). In the normal heart, the amplitude of SR Ca\(^{2+}\) release can be dynamically increased by activation of CaMKII at faster heart rates, leading to a frequency-dependent enhancement of cardiac contractility. In contrast, it also has been suggested that chronic elevation of CaMKII activity in diseased hearts can cause diastolic Ca\(^{2+}\) leak from the SR associated with a loss of contractility. However, it remains unclear which Ca\(^{2+}\) handling proteins downstream of CaMKII are responsible for SR Ca\(^{2+}\) leak abnormalities, although the ryanodine receptor (RyR2) and phospholamban can be functionally altered by CaMKII.

In previous work, we have demonstrated that CaMKII predominantly regulates RyR2 by phosphorylation of residue
S2814.6,8 This site is near, but distinct from, the primary protein kinase A (PKA) phosphorylation site S2808, which also modulates gating properties of the channel.12 We recently demonstrated that CaMKII phosphorylation of RyR2 is sufficient to increase SR Ca\(^{2+}\) leak in mice with constitutively phosphorylated RyR2 attributable to mutation S2814D.6 Moreover, this RyR2-mediated SR Ca\(^{2+}\) leak leads to the development of late-onset cardiomyopathy in S2814D mice, suggesting that chronic phosphorylation of S2814 might promote the development of HF.6 These data are consistent with studies showing that pharmacological or genetic inhibition of CaMKII can prevent or delay the onset of HF in animal models.5,13 However, it also has been shown that PKA hyperphosphorylation of RyR2 at S2808 occurs in failing hearts and that genetic inhibition of S2808 phosphorylation prevents development of ischemic HF in some mouse models.14,15 Therefore, the contribution of these two phosphorylation sites on RyR2 in the development of HF remains controversial.

Our studies revealed increased phosphorylation of S2814 on RyR2 in patients with nonischemic dilated cardiomyopathy (DCM), but not in patients with ischemic cardiomyopathy (ICM). Knock-in mice with a genetically inactivated S2814 phosphorylation site (S2814A mutation) were relatively protected from HF development after transverse aortic constriction (TAC) compared to wild-type (WT) littermates. These effects were associated with a decline in the amount of spontaneous SR Ca\(^{2+}\) release events after TAC in S2814A mice, attributable to prevention of enhanced S2814 phosphorylation. Interestingly, S2814A mice were not protected from the development of ischemic HF after myocardial infarction (MI), consistent with our data obtained in ICM human samples. Thus, our findings suggest that increased CaMKII phosphorylation of RyR2 plays a critical role in the development of pathological SR Ca\(^{2+}\) leak and HF progression in nonischemic forms of HF in both humans and mice with transverse aortic constriction.

Methods
Detailed Methods are provided in the Online Supplement and provide expanded details of surgical procedures, echocardiography, MRI, hemodynamic measurements, histology, Western blot analysis, quantitative reverse-transcriptase polymerase reaction, and calcium imaging.

Surgical Procedures
Generation of RyR2-S2814A knock-in mice has been described.16 All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, conforming to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (National Institutes Health Publication No. 85–23, revised 1996). TAC and MI were performed as described.15,17

Statistical Analysis
All data are represented as average ± SEM. Statistical significance of differences between experimental groups was determined using Student paired t test or ANOVA, followed by Tukey posttest when appropriate. A value of \(P<0.05\) was considered statistically significant.

Results

Increased S2814 Phosphorylation of RyR2 in Patients With Nonischemic Dilated Cardiomyopathy
Increased activity of CaMKII has been suggested to contribute to contractile dysfunction and hypertrophy development associated with HF in patients and in animal models.2,3,18 To determine whether RyR2 phosphorylation by CaMKII is altered in patients with HF, we measured RyR2 phosphorylation levels of S2814 (the principal CaMKII site) as well as S2808 (the principal PKA site and potential secondary CaMKII site) using phosphopeptide-specific antibodies. Ponceau staining confirmed that similar amounts of heart lysate were loaded on the protein gels. Western blots revealed a significant increase in S2814 phosphorylation in nonischemic DCM but not in ICM patients, compared with healthy controls (Figure 1). In contrast, there were no significant changes in S2808 phosphorylation of RyR2 in both patient groups. The finding that S2814 phosphorylation of RyR2 is elevated in patients with DCM was confirmed in a second cohort of nonischemic DCM patients (Online Figure IA, B, and Online Table I). In these samples, increased RyR2 phosphorylation of S2814 may be attributed to enhanced CaMKII activity, because CaMKII T286 autophosphorylation was increased in nonischemic DCM patients compared with controls (Online Figure IC, D).

S2814A Knock-In Mice Exhibit Reduced HF Development in Response to Pressure Overload
Because previous studies have suggested that CaMKII activity plays an important role in development of HF after
pressure overload in mice, we tested whether CaMKII-mediated phosphorylation of RyR2 is important for HF development. We studied RyR2-S2814A knock-in mice (S2814A) in which the CaMKII phosphorylation site was genetically inactivated. At baseline, cardiac dimensions and function in S2814A mice were similar to WT mice up to at least 12 months of age, as determined by echocardiography (Online Table II). Next, 10- to 12-week-old S2814A mice (n=9) and WT littermates (n=13) were subjected to TAC. TAC was performed by partial constriction of the transverse aorta between the right and left carotid arteries, which led to pressure overload. Additional S2814A (n=9) and WT (n=8) mice were subjected to a sham procedure. One-week after TAC, Doppler ultrasound was performed to measure flow velocity in the right and left carotid arteries to estimate the severity of aortic stenosis. The ratio between right and left carotid flow velocities was similar in S2814A (6.9±0.3) and WT mice (6.2±0.2), which indicates that both groups were subjected to similar levels of pressure overload. To determine the effects of the S2814A mutation on development of cardiac hypertrophy and failure, cardiac geometry and function were evaluated using serial echocardiography 0, 4, 8, 12, and 16 weeks after TAC (Figure 2A–C and Table 1).

Echocardiographic analysis revealed a similar hypertrophic response in S2814A and WT mice after TAC, evidenced by a similar initial increase in left ventricular posterior wall thickness compared with sham mice (Figure 2A). There was, however, a trend toward a lower left ventricular posterior wall diameter during diastole at 16 weeks after TAC in WT mice compared with S2814A mice (0.78±0.02 mm versus 0.83±0.04 mm, respectively; P=0.20), suggesting ventricular wall thinning as a possible result of cardiac dilation. WT mice had development of a more pronounced cardiac dilatation after pressure overload in comparison with S2814A mice starting at 12 weeks after surgery (Figure 2B). The ejection fraction declined similarly in S2814A and WT mice up to 8 weeks after TAC (Figure 2C). However, subsequently only WT mice exhibited a further decline in ejection fraction consistent with development of severe HF, whereas ejection fraction leveled off in S2814A mice. At 16 weeks after TAC, ejection fraction was
Table 1. Echocardiographic Parameters of Wild-Type and S2814A Mice at 8 and 16 Weeks After Sham or Transverse Aortic Constriction Surgery

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham 8 Weeks (WT)</th>
<th>S2814A 8 Weeks</th>
<th>Sham 16 Weeks (WT)</th>
<th>S2814A 16 Weeks</th>
</tr>
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<tbody>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>462.1 ± 10.5</td>
<td>467.9 ± 16.1</td>
<td>489.2 ± 12.3</td>
<td>471.3 ± 16.0</td>
</tr>
<tr>
<td><strong>ESD (mm)</strong></td>
<td>2.76 ± 0.08</td>
<td>2.75 ± 0.07</td>
<td>3.36 ± 0.14*</td>
<td>3.12 ± 0.21</td>
</tr>
<tr>
<td><strong>EF (%)</strong></td>
<td>59.2 ± 1.5</td>
<td>58.0 ± 1.0</td>
<td>42.1 ± 2.6‡</td>
<td>43.7 ± 2.2‡</td>
</tr>
<tr>
<td><strong>FS (%)</strong></td>
<td>31.1 ± 1.0</td>
<td>30.2 ± 0.7</td>
<td>20.7 ± 1.4‡</td>
<td>21.3 ± 1.2‡</td>
</tr>
<tr>
<td><strong>IVS, diastolic (mm)</strong></td>
<td>0.75 ± 0.02</td>
<td>0.74 ± 0.01</td>
<td>0.84 ± 0.03</td>
<td>0.77 ± 0.03</td>
</tr>
<tr>
<td><strong>LVPW, diastolic (mm)</strong></td>
<td>0.88 ± 0.02</td>
<td>0.85 ± 0.02</td>
<td>0.94 ± 0.02</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td><strong>LVPW, systolic (mm)</strong></td>
<td>0.95 ± 0.03</td>
<td>0.95 ± 0.03</td>
<td>1.11 ± 0.03*</td>
<td>1.10 ± 0.04*</td>
</tr>
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Data are expressed as mean ± SEM. *P < 0.05 vs corresponding sham. †P < 0.01 vs corresponding sham. ‡P < 0.001 vs corresponding sham. §P < 0.05 vs WT TAC.

significantly higher in S2814A mice (43.0% ± 2.9%) compared with WT mice (32.4% ± 3.4%; P < 0.05; Table 1).

To confirm these results, at 16 weeks after TAC, cardiac contractility also was evaluated using left ventricular catheterization and pressure–volume measurements (Figure 2D, Online Table III).20,21 These hemodynamic measurements revealed a significant decrease in the first derivative of left ventricular pressure over time (dP/dt_max) in WT mice after TAC (6142 mm Hg/s) compared with WT sham mice (10 498 mm Hg/s; P < 0.001), indicating loss of systolic function attributable to pressure overload (Figure 2E). In S2814A mice, however, dP/dt_max was not significantly decreased after TAC (8068 mm Hg/s) compared with sham (8883 mm Hg/s; P > 0.05). Quantification of diastolic blood pressure (P_min or end-diastolic pressure) revealed impaired diastolic function in WT mice after TAC (8.46 mm Hg) compared with sham (8.1 mm Hg; P < 0.01; Figure 2F). Thus, these data suggest that inhibition of RyR2 phosphorylation by CaMKII is sufficient to reduce the development of HF and to preserve cardiac function in a mouse model of nonischemic HF.

### Reduction in Cardiac Dilatation in S2814 Mice After TAC

To further study structural changes in the heart induced by TAC, we performed postmortem analyses of the hearts at 16 weeks postsurgery. Heart weight-to-tibia length (TL) ratios were increased in WT mice (13.8 mg/mm ± 1.4 mg/mm) at 16 weeks after TAC compared with sham-operated WT mice (7.9 mg/mm ± 0.4 mg/mm; P < 0.01; Figure 3A). In contrast, there was a smaller but still significant increase in heart weight/TL ratios of S2814A mice after TAC (9.9 mg/mm ± 0.6 mg/mm) compared with sham hearts (7.6 mg/mm ± 0.5 mg/mm; P < 0.01). In addition, the heart weight/TL ratio was smaller in S2814A compared with WT mice after TAC (Figure 3A).

Hematoxylin and eosin staining of transverse cardiac sections confirmed that S2814A mice exhibited a hypertrophic response (ie, thickening of left ventricular posterior wall) 16 weeks after TAC (Figure 3B). In contrast, WT mice exhibited slightly thinner posterior walls with enlarged cavities (Figure 3C), consistent with cardiac dilatation. Only hearts from WT mice but not S2814A mice exhibited a significant increase in cardiac anteroposterior diameter at 16 weeks after TAC (Figure 3D), consistent with our echocardiography data (Figure 2A–C). Moreover, peak systolic ventricular pressure (end-systolic pressure, mm Hg) was decreased in WT mice (121.1 ± 11.5) compared with S2814A mice (148.7 ± 8.1) after 16 weeks of TAC, consistent with more severe HF development in WT mice after TAC. Correlation of end-systolic pressure values with heart weight/TL ratios revealed less cardiac enlargement despite higher pressures in S2814A mice after TAC, consistent with a protective effect of the S2814A mutation (Online Figure II). Therefore, inhibition of CaMKII-mediated phosphorylation of RyR2 does not prevent the hypertrophic response, but it prevents the progression from hypertrophy to cardiac dilatation and failure in the setting of pressure overload.

### Reduced Cardiac Remodeling Associated With HF After TAC in S2814A Mice

To determine whether genetic inhibition of CaMKII phosphorylation of RyR2 prevented adverse remodeling of the heart after TAC, Masson trichrome staining of cardiac sections was performed to measure cardiac fibrosis (Figure 4A, B). Whereas there was a significant increase in the development of cardiac fibrosis in WT mice after TAC, the amount of fibrosis was reduced in S2814A mice after TAC (Figure 4B). Additionally, WT mice had development of a significant increase in lung weight-to-TL ratio after TAC (14.6 mg/mm ± 1.7 mg/mm) compared with sham-operated mice (9.5 mg/mm ± 0.4 mg/mm;
which is indicative of pulmonary edema in the context of congestive HF (Figure 4C). In contrast, there was no significant increase in lung weight/TL ratio after TAC in S2814A mice (9.4 mg/mm$^2$ ± 0.3 mg/mm) compared with sham-operated S2814A mice (9.9 mg/mm$^2$ ± 0.6 mg/mm). However, at 16 weeks after TAC, there was a significant increase in lung weight/TL ratio in WT compared with S2814A mice, suggesting a rescue from pulmonary edema development in S2814A mice (Figure 4C).

Another major determinant of stress-induced cardiac remodeling is reactivation of fetal cardiac genes. Therefore, mRNA levels of fetal genes nppa (atrial natriuretic factor) and nppb (brain natriuretic peptide) were determined using quantitative polymerase chain reaction. In sham-operated animals, there were no differences in transcript levels comparing S2814A and WT mice. In contrast, TAC induced a significant increase in atrial natriuretic factor and brain natriuretic peptide levels in WT mice, compared with sham-operated WT mice (Figure 4D, E). However, there was no increase in atrial natriuretic factor levels and a blunted brain natriuretic peptide response in S2814A mice compared with WT mice after TAC. Taken together, these data suggest that inhibition of CaMKII phosphorylation of RyR2 does not suppress the hypertrophic response after pressure overload, but it does prevent cellular signs of maladaptive HF.

Increased S2814 but Not S2808 Phosphorylation of RyR2 After TAC

Next, we determined the time course of potential changes in RyR2 phosphorylation at S2814 (the principal CaMKII site) and S2808 (the principal PKA site). Western blotting of ventricular lysates using phosphoepitope-specific antibodies revealed an increase in CaMKII phosphorylation of S2814 on RyR2 in WT mice after TAC (Figure 5A). The level of S2814 phosphorylation displayed a gradual increase, which became significant at 8 and 16 weeks after TAC (Figure 5B). As expected, there was no phosphorylation of this site in S2814A mice because of the
Inhibition of CaMKII Phosphorylation of RyR2 Attenuates SR Ca\(^{2+}\) Leak After TAC

To determine the mechanisms underlying sustained cardiac function in S2814A mice after TAC, we next determined whether inhibition of CaMKII-mediated phosphorylation of RyR2 attenuated spontaneous SR Ca\(^{2+}\) release (SCR) events after pressure overload. Ventricular myocytes isolated from mice at 16 weeks after TAC or sham surgery were loaded with a Ca\(^{2+}\)-sensitive dye and imaged under an epifluorescence microscope. In previous studies, we found a good correlation between SR Ca\(^{2+}\) leak measured using the tetracaine protocol and the number of SCR events. After 1 Hz pacing to obtain steady-state, the number of SCR events was measured after termination of pacing over a 40-second time period (Figure 6A). The number of myocytes in which SCR events occurred was significantly higher in WT mice after TAC (46 events in 75 cells; ≈61%) compared with WT sham mice (8 events in 31 cells; ≈26%; P < 0.01; Figure 6B). In addition, SCR amplitude (measured as ΔF/ΔFo) was also increased in WT TAC compared with WT sham mice. In contrast, SCR amplitude was not increased in S2814A TAC compared with S2814A sham mice (Online Figure IVA). The increase in SCR was not attributable to increased SR Ca\(^{2+}\) loading, because SR content was decreased in WT TAC (ΔF/ΔFo: 1.54±0.1) compared with WT sham mice (1.97±0.2; P < 0.05; Online Figure IVB). Moreover, the increase in SCR amplitude also was not attributable to increased SR Ca\(^{2+}\) content, because each release event was normalized to SR Ca\(^{2+}\) content (Online Figure IVC).

There were no differences in the rate of uptake of Ca\(^{2+}\) into the SR, a measure of SERCA activity (tau [τ]; Online Figure IVD). The incidence of SCR in myocytes from S2814A mice after TAC (29.7%) was not increased compared with sham-operated S2814A mice (29.4%) and was significantly lower than myocytes from WT mice after TAC (61.3%; P < 0.01; Figure 6B). Moreover, SR Ca\(^{2+}\) content remained unchanged in S2814A mice after TAC (1.88±0.1) and S2814A sham mice (1.96±0.2; Online Figure IVB). These data suggest that the increase in SCR incidence in WT mice after TAC is attributable to increased S2814 phosphorylation on RyR2 induced by TAC.

Similar findings were obtained when the average number of SCR was quantified per myocyte (Figure 6C). In WT mice, there was a significant increase in SCR events per myocyte over a 40-second time period after TAC (2.0±0.3 events/cell) in comparison with WT sham (0.4±0.1; P < 0.01). This increase in SCR event rate attributed to TAC was blunted in S2814A mice (0.4±0.1; P < 0.01). Finally, addition of the global CaMKII inhibitor KN93 also significantly reduced the SCR incidence and number of SCR events per cell in myocytes from WT mice after TAC (Figure 6B, C). However, KN93 had no additional effect on SCR incidence or events in S2814A TAC cells, suggesting a specific and major role for S2814 phosphorylation on RyR2 in the development of HF.

Inhibition of S2814 Phosphorylation on RyR2 Fails to Protect Against HF Induced by MI

Previous studies demonstrated that PKA phosphorylation of RyR2 played a role in the development of ischemic HF after MI, but not after TAC-induced pressure overload. However, these data are controversial in view of a recent study that argued against a role for PKA phosphorylation of RyR2 after MI. To determine the functional importance of CaM-
KII phosphorylation of RyR2 during the development of ischemic HF, we subjected S2814A knock-in and WT mice to left anterior descending coronary artery ligation to induce MI. In mice subjected to the sham procedure, cardiac dimensions and contractility (Online Table IV) were similar in S2814A mice compared with WT mice. The effects of both mild (30% infarct area) and severe (60% infarct area) MI were determined in two separate experimental groups of S2814A and WT mice (Figure 7A, B), as determined by MRI and echocardiography and confirmed by histology. Infarct size did not differ in the 30% group comparing S2814A (32.8%±2.2%) and WT mice (32.4%±1.0%) or in the “60% group” comparing S2814A (61.8%±2.1%) and WT (64.9%±4.0%). Echocardiography revealed that there were no differences in the relative increase in left ventricular end-diastolic diameter comparing S2814A and WT mice after both 30% and 60% MI 3 weeks after surgery (Figure 7A). As expected, cardiac dilatation was more pronounced after 60% infarction compared with 30% infarction. Similarly, there was an equal decline in ejection fraction in S2814A and WT mice after both 30% and 60% MI (Figure 7B). There were no significant differences in ejection fraction comparing S2814A and WT mice. At the end of the experiment, ventricular myocytes were isolated from the sham and 60% MI mice. At 3 weeks after coronary artery ligation, ventricular myocyte length was increased similarly in S2814A and WT mice (Figure 7C).

To determine the levels of CaMKII-mediated RyR2 phosphorylation in response to MI, we performed Western blot analysis of ventricular lysates obtained from mice subjected to 60% MI. Using phospho-specific antibodies, the level of S2814 phosphorylation on RyR2 was significantly decreased in WT mice (P<0.001). As expected, there was no phosphorylation of S2814 in S2814A mice attributable to the genetic mutation of this site (Online Figure VA, B). Taken together, these data suggest that inhibition of CaMKII phosphorylation on S2814 of RyR2 does not prevent the development of HF induced by MI.

Discussion

There is ample evidence that CaMKII plays a role in the development of HF. Previous studies have demonstrated increased expression and activity levels of CaMKII in animals and patients with congestive HF.10,26,27 Transgenic overexpression of CaMKII-δ causes HF in mice,3 whereas overexpression of a peptide blocker of CaMKII delays the onset of HF in AC3-I transgenic mice.4 Moreover, knockout of CaMKII-δ was shown to limit the progression to HF.13,18 It

31 34 75 37 61 30

30 to 75 cells from 3 to 6 mice per group. **P<0.01 vs corresponding sham. ###P<0.01 vs wild-type TAC.

Figure 6. Inhibition of CaMKII phosphorylation of RyR2 attenuates sarcoplasmic reticulum (SR) Ca2+ leak after transverse aortic constriction (TAC). A, Representative images of spontaneous Ca2+ release (SCR) events in ventricular myocytes after 1-Hz pacing. B, Incidence of SCR events in isolated myocytes from sham-operated and mice 16 weeks after TAC, before and after pretreatment with KN93, respectively. C, Quantification of SCR events per cell before and after pretreatment with KN93. N=30 to 75 cells from 3 to 6 mice per group. **P<0.01 vs corresponding sham. ###P<0.01 vs wild-type TAC.

Figure 7. Inhibition of S2814 phosphorylation on RyR2 fails to protect against heart failure induced by myocardial infarction (MI). Echocardiographic analysis of wild-type (WT) and S2814A mice subjected to sham operation or MI, resulting in average infarcts comprising 30% or 60% of the left ventricle, respectively. There were no differences in left ventricular end-diastolic diameter (LVEDD) (A) or ejection fraction (EF) (B) comparing WT and S2814A. Numbers in bars indicate number of animals and cells per group. *P<0.05 vs corresponding sham.
was shown that CaMKII-δ ablation reduced ventricular dilatation and fibrosis and enhanced cardiac contractility after TAC.13 The beneficial effects of CaMKII-δ ablation have been attributed to a reduction in SR Ca2+ leak, although the downstream targets of CaMKII responsible for these effects were not identified.

The results of the current study revealed increased CaMKII phosphorylation of RyR2 in patients with nonischemic DCM but not in patients with ICM. These findings suggest that the level of CaMKII activation might depend on the type of HF in patients. Previous studies have clearly demonstrated activation of CaMKII-δ by pressure overload in mice.13,18,28 Our data in mice showed that S2814 phosphorylation on RyR2 is increased in mice subjected to TAC (pressure overload) but not after MI. An important observation was that S2814 phosphorylation increases over time after TAC in WT mice, whereas ablation of the S2814 site alone provided a beneficial effect on deterioration toward severe HF in these mice. Thus, our data now identify RyR2 as a downstream target of CaMKII involved in HF after pressure overload.

Moreover, our data for mice subjected to TAC revealed increased CaMKII phosphorylation of phospholamban at T17, but not at the PKA site S16. These findings suggest that activated CaMKII phosphorylates multiple downstream targets in failing hearts. However, the protective effects of the S2814A mutation in RyR2 in mice subjected to TAC shows that RyR2 is a preeminent downstream target of CaMKII in this model of HF associated with detrimental remodeling of the heart. Thus, RyR2 phosphorylation at S2814 plays a role in the progression of cardiac hypertrophy to HF after TAC.

Transgenic overexpression of CaMKII-δ induces transient cardiac hypertrophy followed by dilated cardiomyopathy and HF in mice.3 However, hypertrophic remodeling induced by pressure overload was not attenuated in CaMKII-δ-deficient mice, suggesting that CaMKII-δ is not required for development of cardiac hypertrophy in response to TAC.13 Our data are in agreement with those previous studies of CaMKII-δ-deficient mice, because S2814A mice also had development of hypertrophic remodeling similar to that of WT mice in the early stages after TAC.

Effects of CaMKII Phosphorylation of RyR2 on SR Ca2+ Leak in HF

Enhanced activity of CaMKII in the heart profoundly affects SR Ca2+ handling.2 Ventricular myocytes isolated from CaMKII-δc transgenic mice exhibit increased diastolic SR Ca2+ release events (SR Ca2+ leak) despite a lower SR Ca2+ load,2 which could be partly caused by increased CaMKII phosphorylation of RyR2. Phosphorylation of the CaMKII phosphorylation site S2814 on RyR2 enhances channel open probability and Ca2+ spark activity.6,8 Our recent studies revealed that constitutive activation of this CaMKII site in S2814D knock-in mice causes SR Ca2+ leak, which is associated with a mild dilated cardiomyopathy at 12 months of age.6 Moreover, S2814D mice exhibit markedly reduced survival after TAC, suggesting that maximal phosphorylation of S2814 on RyR2 promotes development of decompensated HF.6

Our data revealed an increased incidence of spontaneous SCR events in WT mice after TAC. Pharmacological inhibition of CaMKII reduced the number of SCR events, suggesting that CaMKII activation after pressure overload underlies Ca2+ release defects, as previously shown.2,13 Genetic ablation of the S2814 phosphorylation site on RyR2 led to a similar decrease in SCR incidence after TAC in the absence of exogenous CaMKII blockade, suggesting that RyR2 is a
major downstream target of CaMKII underlying defective SR Ca\(^{2+}\) release. Thus, pressure overload induces spontaneous releases of SR Ca\(^{2+}\) during diastole attributable to increased CaMKII-mediated phosphorylation of RyR2. Taken together, these data suggest that the preservation of cardiac contractility, attenuation of SR Ca\(^{2+}\) leak, and delayed development of HF in S2814A mice are mediated by the direct prevention of CaMKII phosphorylation of site S2814 on the RyR2 Ca\(^{2+}\) release channel.

**Conclusions**

Taken together, our present work has demonstrated that increased CaMKII phosphorylation of S2814 on RyR2 plays a critical role in the development of pathological SR Ca\(^{2+}\) leak and HF in a mouse model of pressure overload. However, phosphorylation of the S2814 site does not appear to play a role in the development of HF after MI. Moreover, there seems to be a difference in phosphorylation of RyR2 sites depending on whether HF is ischemic in nature. Overall, our current findings suggest an important role for CaMKII-mediated phosphorylation of S2814 on RyR2 in the development of nonischemic HF in mice with pressure overload, and support its role as a potential new target for the treatment of HF.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Heart failure (HF) is a leading cause of morbidity and mortality and is responsible for one of every nine deaths in the United States.
- Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is upregulated in patients with HF.
- Phosphorylation of CaMKII phosphorylation site S2814 on RyR2 enhances open probability, Ca\(^{2+}\) spark activity, and sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak.

**What New Information Does This Article Contribute?**

- S2814 phosphorylation of RyR2 is increased in patients with nonischemic dilated cardiomyopathy (DCM) and in mice after pressure overload, but not in patients and mice with ischemic cardiomyopathy (ICM).
- Genetic inhibition of S2814 phosphorylation in mice was found to prevent development of decompensated HF after TAC. The time course of the protective effect correlated well with the delayed increase in S2814 phosphorylation on RyR2. Finally, SR Ca\(^{2+}\) leak was reduced in S2814A knock-in mice after TAC, compared with WT control mice. In contrast, S2814A mice were not protected from development of HF after myocardial infarction, consistent with the findings in human ICM samples. Thus, our data suggest that increased S2814 phosphorylation of RyR2 might play an important role in development of nonischemic types of HF.

Previous studies demonstrated that increased levels of CaMKII might contribute to enhanced SR Ca\(^{2+}\) leak in animals and patients with HF. Here, we tested the hypothesis that CaMKII phosphorylation of S2814 on RyR2 underlies Ca\(^{2+}\) cycling defects in HF. First, we found that S2814 phosphorylation is only increased in patients with nonischemic DCM, but not in those with ICM. Next, genetic inhibition of S2814 phosphorylation in mice was found to prevent development of decompensated HF after TAC. The time course of the protective effect correlated well with the delayed increase in S2814 phosphorylation on RyR2. Finally, SR Ca\(^{2+}\) leak was reduced in S2814A knock-in mice after TAC, compared with WT control mice. In contrast, S2814A mice were not protected from development of HF after myocardial infarction, consistent with the findings in human ICM samples. Thus, our data suggest that increased S2814 phosphorylation of RyR2 might play an important role in development of nonischemic types of HF.