Phosphorylation of RyR₂ and shortening of RyR₂ cluster spacing in spontaneously hypertensive rat with heart failure

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DURING THE DEVELOPMENT of hypertensive heart disease, heart failure (HF) is often preceded by concentric cardiac hypertrophy (11). Although the molecular mechanisms of this transition are incompletely understood (9), defects in excitation-contraction (E-C) coupling are thought to play a central role in cardiac hypertrophy and HF (12, 43). In HF, increased phosphorylation of ryanodine receptors (RyR₂) at Ser²⁸⁰₈ in the spontaneously hypertensive rat (SHR) at four distinct disease stages. A major finding is that hyperphosphorylation of RyR₂ at Ser²⁸⁰₈ occurred only at late-stage heart failure in SHR, but not in age-matched controls. Furthermore, the spacing between RyR₂ clusters was shortened in failing hearts, as predicted by quantitative model simulation to increase spontaneous Ca²⁺ wave generation and arrhythmias.

The progression of concentric hypertrophy to HF has been well demonstrated in the spontaneously hypertensive rat (SHR) model, which mimics human essential hypertension and heart disease (4). We hypothesized that in the SHR model the transition from cardiac hypertrophy to HF might influence the level of RyR₂ phosphorylation, as well as the subcellular organization of CRUs. The purpose of this study is to determine whether the progressive development of hypertensive heart disease in SHR is accompanied by 1) changes in the phosphorylation of RyR₂-Ser²⁸⁰₈ and 2) changes in the spatial distribution of CRUs in ventricular myocytes.

MATERIALS AND METHODS

Animals and tissue preparation. Male SHR, normotensive Wistar-Kyoto rats (WKY), and Sprague-Dawley rats were purchased from Charles River (http://www.criven.com). Blood pressure was monitored weekly in nonanesthetized SHR and WKY by the tail-cuff method. For tissue preparation, rats were anesthetized with pentobarbital sodium (100 mg/kg ip) with 4,000 U/kg heparin. Isoproterenol (1 mg/kg ip) was injected before cardiac explantation for the β-adrenergic receptor stimulation experiments. After suppression of spinal cord reflexes, the heart was exposed via a midline thoracotomy, and

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the chambers were rinsed with Ca\(^{2+}\)-free PBS, which was injected into the left ventricle (LV) and vented through a small incision in the right atrial free wall. The heart was subsequently removed, and laterally dissected to expose the ventricular walls and chambers. Tissue freezing medium (OCT compound) was injected into the chambers to preserve cardiac morphology during the subsequent freezing process. The tissue was flash frozen by submersion in chilled isopentane for 10–20 s, placed on dry ice, and then stored in a -80°C freezer. The frozen tissue was cut into 20-µm-thick sections in a cryostat (model 2800 Frigocut-E, Reichert, Bannockburn, IL). All chemicals and reagents were purchased from Sigma-Aldrich if not specified otherwise. All animals were handled strictly in accordance to the National Institutes of Health guidelines and protocols approved by our Institutional Animal Care and Use Committee at the University of Kentucky.

**Western blot.** Western blot was used to measure the amount of phosphorylation of RyR2-Ser\(^{2808}\) relative to the total amount of RyR2 in LV tissue according to a previously published protocol (32). Phosphorylated RyR2-Ser\(^{2808}\) (RyR2-pSer\(^{2808}\)) was stained using a phosphorylated epitope-specific antibody [affinity-purified polyclonal rabbit antibody raised against phosphorylated peptide sequence C-TRRRRI(pS)-GSQTV]; total RyR2 was stained using a polyclonal anti-RyR2 antibody.

**Semiquantitative immunohistochemistry.** Tissue sections were incubated in a blocking solution containing 5% goat serum and 3% BSA in PBS for 30 min and rinsed twice in PBS; then the sections were incubated in primary antibody solution (1:200 dilution) for 1.5 h, rinsed twice in PBS, and incubated in secondary antibody solution (1:200 dilution) for 1.5 h. The same phosphorylated epitope-specific (polyclonal, rabbit) antibody was used in semiquantitative immunohistochemistry (SQ-IHC) and quantitative Western blot to label RyR2-phosphorylation. Phosphorylated and pan RyR2 labeling were visualized using secondary antibody-conjugated fluorophores: anti-rabbit IgG conjugated Alexa Fluor 568 and anti-mouse IgG-conjugated Alexa Fluor 488, respectively. The antibody-labeled tissue sections were covered with Antifade and sealed under a glass coverslip (no. 1) for imaging.

**Confocal imaging and image analysis for SQ-IHC.** Confocal images were obtained using a confocal microscope (Radiance 2000, Bio-Rad) with a water immersion objective (×63, NA 1.2) corrected for the thickness of the no. 1 glass coverslip. To obtain confocal images of antibody labeling, we placed the focal plane in the middle of the 20-µm-thick tissue slice to avoid the interface between the tissue and the glass. The imaging areas were chosen by random scanning of the tissue section, and various areas in the tissue section were used to obtain an average value. For quantitative analysis and comparison between the control group and the test group, we strictly used identical antibody labeling conditions and confocal imaging parameters for all the tissue samples in the groups of comparison.

To quantitatively measure the labeling intensity, we recorded the fluorescence emission from a defined area (using a ×63 objective and 1,024 × 1,024 pixels in the x-y plane) with a defined depth (pinhole size optimized to confocal z resolution of ∼1 µm). Histograms of the optical intensity [i.e., fluorescence intensity (FI)] of each pixel were plotted. The background signal was subtracted from the total histogram before the average FI was calculated. The average FI was then calculated for each image. To calculate the relative phosphorylation of RyR2-Ser\(^{2808}\), we double labeled RyR2-Ser\(^{2808}\) and the total RyR2 in the tissue using specific primary antibodies and secondary antibody-conjugated Alexa Fluor 568 and Alexa Fluor 488, respectively. The ratio of the average FI of Alexa 568 to that of Alexa 488 reflects the amount of RyR2-Ser\(^{2808}\) relative to the total amount of RyR2.

The images used to calculate the antibody labeling intensity were obtained without digital zoom (gain = 1), and the images used to measure the spacing between RyR2 clusters (CRUs) were obtained with maximum digital zoom (gain = 10). The spacing between RyR2 clusters along the longitudinal axis of the cells was measured using our previously described method (7).

**Results.** The rats were anesthetized with pentobarbital sodium (Nembutal; 100 mg/kg ip). Hearts were tested for the suppression of reflexes and then explanted via a midline thoracotomy. A standard enzymatic technique was used to isolate the ventricular myocyte. Briefly, the heart was mounted on a Langendorff system and perfused with a modified Tyrode solution containing (in mmol/l) 135 NaCl, 4 KCl, 1.0 MgSO\(_4\), 0.34 NaH\(_2\)PO\(_4\), 15 glucose, 10 HEPES, and 0.1 taurine, with pH adjusted to 7.25 with NaOH; the perfusion solution was prewarmed to 37°C and bubbled with 100% O\(_2\). Then collagenase B (~1 mg/ml; Hoffmann-La Roche, Basel, Switzerland), protease type XIV (~0.1 mg/ml), 0.1% BSA, and 20 mM Ca\(^{2+}\) were added into the perfusion solution, and the heart was enzymatically digested for 15–20 min. The ventricular tissue was cut down and minced, the remaining tissue was further incubated in the enzyme solution at 37°C for 15–45 min and minced again, and the ventricular myocytes were collected.

**Immunocytochemistry.** Freshly isolated ventricular myocytes were labeled using anti-RyR2 monoclonal antibody (mouse IgG1; clone C3-33, Affinity BioReagents) as described precisely in detail (7, 14).

**Statistical analysis.** All samples were coded using randomized codes for blinded processing during experiments and data analysis. Values are means ± SD. Unpaired Student’s t-test with equal variance and two tails was used to compare the age-matched SHR vs. WKY; the difference in the mean values is deemed significant if P < 0.05. Two-way ANOVA was used to evaluate the differences in the longitudinal study of the age-related changes in WKY and in the disease-related changes in SHR.

**RESULTS**

**Progressive development of hypertrophy and HF in SHR.** Hypertensive heart disease develops progressively in SHR through distinctive stages of hypertension, cardiac hypertrophy, and HF. The history of blood pressure development is shown in Fig. 1A. SHR were prehypertensive during their first 4–6 wk of life, rapidly developed hypertension at 8–12 wk of age, and remained hypertensive thereafter. In comparison, WKY controls remained normotensive throughout their ~2-yr life span. After the onset of hypertension, cardiac hypertrophy gradually developed in SHR at ~3–18 mo of age, and HF typically occurred at 1.5–2 yr of age. The history of hypertrophy development in SHR is shown in Fig. 1B. As a normotensive control, WKY displayed a slight decrease in heart weight-to-body weight ratio (HW/BW), as the increase in body weight outpaced the increase in heart weight as the animals aged. HW/BW was the same in 5-wk-old (prehypertensive) and 11-wk-old (onset of hypertension) SHR as in age-matched WKY controls. HW/BW was significantly higher in 1-yr-old SHR than in age-matched WKY, demonstrating cardiac hypertrophy: 5.38 ± 0.77 (n = 10) vs. 3.84 ± 0.51 (SD) mg/g (n = 7; P < 0.05, t-test). During HF, HW/BW was higher in SHR than in age-matched WKY [6.19 ± 1.68 (n = 5) vs. 3.63 ± 1.13 mg/g (n = 18)], but the individual variation was greater than in age-matched SHR. Most likely due to cardiac cachexia (P < 0.1, t-test), consistent with previous studies showing echocardiographic evidence for HF in >1.5-yr-old SHR (6, 8, 34, 35), we found clinical signs of HF (e.g., severe hypertrophy or chamber dilation, ascites, pericardial effusion, chest edema, and lung edema) in SHR at postmortem examination.
The results show that RyR$_2$-Ser$^{2808}$ phosphorylation drastically increased in >1.5-yr-old SHR [3.00 ± 0.94 in SHR vs. 1.44 ± 0.49 in WKY (n = 4, P < 0.05 by t-test and $P < 0.001$ by 2-way ANOVA with Bonferroni’s posttest)] after the progression from hypertrophy to HF.

**SQ-IHC method development.** To provide another independent measure for RyR$_2$-Ser$^{2808}$ phosphorylation levels, we developed an SQ-IHC method by combining immunohistochemistry and confocal microscopy techniques (Fig. 2A). The specificity of antibody labeling was verified by preincubation of the antibody with its phosphorylated epitope peptide, which also served as the background image for subtraction of the nonspecific labeling. Basal RyR$_2$-pSer$^{2808}$ in the control tissue was relatively low (Fig. 2B). In contrast, intense labeling of RyR$_2$-pSer$^{2808}$ was observed in cardiac tissue obtained from the heart pretreated with isoproterenol, which induced PKA phosphorylation of RyR$_2$ due to $\beta$-adrenergic stimulation (Fig. 2B). The punctate staining in a striated pattern is also consistent with the known RyR$_2$ clustering and localization on Z disks (7).

The original SQ-IHC method used a digital camera to image and quantify the molecules in tissue samples (25). We improved on the original SQ-IHC method by employing confocal microscopy to image optical sections inside the tissue (avoiding tissue surface artifacts) and strictly controlled the amount of fluorescence emission by using a fixed area and a fixed depth in the optical section [providing normalization for averaging across different sections, so the labeling intensity in each image is measured from a unit volume (normalized) and, hence, can be used to compare different tissue sections]. FI values of all pixels in a representative image are shown in Fig. 2C. The background image showed very low (<34) FI, in accordance with low background labeling. The antibody labeling image showed well-distributed FI values across the digitizing range for intensity without saturation. To subtract the background, we truncated the FI histogram of each image by removing all points <34 FI. This subtraction also digitally removed “holes” in the tissue section caused by physiological structures (e.g., capillary, nerve, and extracellular space). The mean FI of each image was then calculated from the background-subtracted histogram. We used identical treatment for all tissue samples throughout the process, from antibody labeling (reagents and protocol) to confocal imaging (optical and parameter settings), to eliminate variations.

To measure the RyR$_2$-pSer$^{2808}$ relative to the total amount of RyR$_2$ protein in a tissue section, we double labeled sections using a phosphorylated epitope-specific antibody recognizing RyR$_2$-pSer$^{2808}$ and a pan RyR$_2$ antibody tagged with secondary antibody-conjugated Alexa Fluor 568 and Alexa Fluor 488, respectively (Fig. 2D, pseudocolored red for RyR$_2$-pSer$^{2808}$ and green for pan RyR$_2$). The SQ-IHC method was used to obtain mean FI values for each antibody labeling; then we calculated the ratio of mean FI values of RyR$_2$-pSer$^{2808}$ to the total RyR$_2$ labeling, which provided a measure for the relative amount of RyR$_2$-pSer$^{2808}$ in the tissue. A great benefit of this ratiometric method is that it eliminates the variations in different tissue regions (e.g., cell density, orientation, and accessibility) and, hence, enables us to average across different tissue sections. The ratiometric method was used to measure relative RyR$_2$-pSer$^{2808}$ in control and epinephrine-treated hearts, and the results are shown in Fig. 2E. Infusion of epinephrine into
the heart caused β-adrenergic stimulation and increased PKA phosphorylation of RyR2-Ser^{2808}.

The advantage of the SQ-IHC method is that it bypasses several protein-processing steps of the Western blot method, including tissue homogenization, protein extraction, total protein calibration, and protein transfer from gel to membrane, which should help reduce potential artifacts associated with these procedures. The disadvantage of the SQ-IHC method is...
that molecular weights cannot be separated and very specific antibodies are required. Although we treated all samples using identical conditions throughout the antibody labeling and image acquisition procedures and employed the ratiometric method, the SQ-IHC measurement remains semiquantitative. However, the SQ-IHC method can be used in combination with Western blot to verify the data.

**SQ-IHC measurement of RyR2-Ser²⁸⁰⁸ phosphorylation.** Sample images depicting double labeling of RyR2-pSer²⁸⁰⁸ and total RyR2 in the SHR failing heart and age-matched WKY control are shown in Fig. 2D. Although total RyR2 labeling (green) was comparable in SHR and WKY, RyR2-pSer²⁸⁰⁸ labeling (red) was more intense in SHR than WKY, demonstrating an increase in RyR2-pSer²⁸⁰⁸ relative to total RyR2 in SHR.

Relative RyR2-Ser²⁸⁰⁸ phosphorylation in various regions of the heart at three distinct stages of heart disease is shown in Fig. 3. In 11-wk-old SHR at the onset of hypertension before hypertrophy, RyR2-pSer²⁸⁰⁸ levels were similar to those in age-matched WKY across LV, septum, and right ventricle (RV; Fig. 3A; \( P > 0.05 \) for strain difference at each region (\( t \)-test) and \( P > 0.05 \) for strain difference across all regions (2-way ANOVA)). In 1-yr-old SHR with overt cardiac hypertrophy, RyR2-pSer²⁸⁰⁸ remained normal in LV and septum but was significantly increased in RV [Fig. 3B; \( P = 0.01 \) for strain difference across all regions (2-way ANOVA) and \( P < 0.05 \) for RV (Bonferroni’s posttest)]. In >1.5-yr-old SHR during HF, however, RyR2-pSer²⁸⁰⁸ was significantly increased across all regions of the heart, including LV, septum, and RV [Fig. 3C; \( P = 0.002 \) for strain difference across all regions (2-way ANOVA) and \( P > 0.05 \) possibly due to small sample numbers (Bonferroni’s posttest)]. Interestingly, RyR2-pSer²⁸⁰⁸ was significantly higher in LV in >1.5-yr-old SHR \( (P < 0.05, t \)-test) but higher in RV 1-yr-old SHR \( (P < 0.05 \ t \)-test) and \( P < 0.05 \) (Bonferroni’s posttest)] than in age-matched WKY controls. Figure 3D shows the relative RyR2-pSer²⁸⁰⁸ levels in SHR normalized to age-matched WKY. The changes in LV measured using the SQ-IHC method are in agreement with those measures using Western blot (Fig. 1C).

**SQ-IHC measurement of spatial distribution of RyR2 clusters.** The SQ-IHC method enabled us to measure not only the phosphorylation levels of RyR2 but also the spatial localization of RyR2 clusters (each contains \( \sim 100 \) RyR2 molecules and serves as a CRU). We used a previously described method (7) (spatial resolution \( \sim 0.25 \mu m \)) to measure the longitudinal spacing between the neighboring RyR2 clusters in the tissue cross sections (Fig. 4A). The histogram of CRU spacing showed a Gaussian distribution (Fig. 4B). The shorter CRU spacing in the failing heart of the SHR is manifested by a leftward shift of the distribution from that of WKY. To confirm the change of CRU spacing in the failing heart, we also used 2-mo-old healthy Sprague-Dawley rats as an additional control. As shown in Fig. 4C, CRU spacing is 1.72 \( \pm 0.34 \) (SD) \( \mu m \) in the failing heart of the SHR, 9.5% shorter than in the age-matched WKY \( (1.90 \pm 0.32 \mu m, P < 0.01 \ t \)-test) and 11.3% shorter than in the Sprague-Dawley control \( (1.94 \pm 0.31 \mu m, P < 0.01 \) ). Since increased collagen expression in failing hearts (40) might constrain the cell length or shrink the tissue more on processing, we used collagenase and protease to enzymatically digest the heart, to isolate ventricular myocytes. We conducted immunocytochemistry experiments to label...
Furthermore, average CRU spacing in WKY and Sprague-Dawley control hearts is consistent with normal resting sarcomere length in ventricular myocytes (1.8–2.2 μm; data not shown), which demonstrates preserved cellular ultrastructure in the tissue section. (Since RyR2 are localized in Z disks, the longitudinal spacing between CRUs corresponds to the sarcomere length.) Given that the identical protocol was used to prepare all tissues, the shorter CRU spacing in failing hearts of SHR cannot be attributed to artifacts that might change the cellular ultrastructure or to differential collagen expression. Therefore, it is most likely that shortening of the CRU longitudinal spacing in the failing heart is a real phenomenon of pathological significance.

**DISCUSSION**

Hyperphosphorylation of RyR2-Ser2808 after progression from hypertrophy to HF in SHR. In the present study, we investigated two important factors that may contribute to changes in Ca2+ signaling during cardiac hypertrophy and HF: 1) the phosphorylation status of the cardiac RyR (Ser2808) and 2) the spatial distribution of RyR2 clusters within the cardiomyocytes. First, we conducted a longitudinal study to measure RyR2-pSer2808 in SHR at the four distinct stages in the development of hypertensive heart disease: prehypertension, hypertension before hypertrophy, cardiac hypertrophy, and finally HF. Western blot studies revealed that RyR2-pSer2808 in the LV of SHR was unaltered during the prehypertrophic and hypertrophic stages of hypertensive heart disease and became significantly elevated during HF. Immunohistochemistry studies further showed that RyR2-pSer2808 was normal during the prehypertrophic stage of hypertensive heart disease, whereas RyR2-pSer2808 became elevated in the RV of SHR with cardiac hypertrophy. After the development of overt HF in SHR, RyR2-pSer2808 significantly increased across the LV, septum, and RV in >1.5-yr-old rats. Overall, there was good agreement between the data obtained using Western blot and SQ-IHC. Hence, a major finding of this study is that hyperphosphorylation of RyR2-Ser2808 occurs late during the development of hypertensive heart disease, after the development of cardiac failure.

Our data provide new insights into the controversy in the literature regarding increased phosphorylation of RyR2-Ser2808 in structural heart disease. The finding that phosphorylation of RyR2-Ser2808 is only increased in the LV of SHR with overt HF suggests that this posttranslational modification of the RyR may occur late during the development of HF. Our findings are consistent with enhanced PKA phosphorylation of RyR2 in patients with end-stage HF (24, 31). Hyperphosphorylation of RyR2 has also been reported in several animal models of HF, including a canine model of pacing-induced HF (30, 49), a rabbit aortic-banding model (1), and rat (26, 31) and mouse models of ischemic HF (45). Nonetheless, other studies found no changes in RyR2-pSer2808 phosphorylation in patients and animals with HF. In light of the findings of our studies in SHR, it is likely that the degree of RyR2-Ser2808 phosphorylation varies with the etiology and severity of heart disease. Indeed, Ward et al. (41) reported that SR Ca2+ load and RyR2 function were not affected in SHR with mild symptoms of HF.

Fig. 4. Decreased spacing between RyR2 Ca2+ release units (CRUs) in failing hearts of SHR. A: confocal images depicting representative RyR2 labeling in tissue sections from SHR failing hearts and age-matched WKY control (pseudocolored red to distinguish from SHR). Yellow bars depict definition of CRU longitudinal spacing. Scale bars, 2 μm. B: normal distribution of CRU longitudinal spacing in SHR failing hearts (n = 66 determinants from 3 hearts) and WKY controls (n = 156 from 3 hearts). Distribution of spacing in SHR is leftward shifted from that of WKY: mean and variance from Gaussian fit of 1.72 ± 0.34 μm in SHR and 1.90 ± 0.32 μm in WKY (P < 0.001). C: CRU longitudinal spacing in isolated ventricular myocytes (cell) from Sprague-Dawley (SD) controls (n = 786 determinants) and SHR failing hearts (n = 130) and in tissue cross sections (tissue) from Sprague-Dawley (n = 19) and WKY controls (n = 156) and SHR failing hearts (n = 66). Values are means ± SD.

RyR2 clusters in isolated cells and measured the longitudinal spacing between the antibody-labeled CRUs (Fig. 4C). Again, CRU spacing was shorter in cells from the failing heart of the SHR (1.77 ± 0.15, n = 130) than in cells from the Sprague-Dawley control [1.87 ± 0.18, n = 786, P < 0.001 (t-test)].
Factors that regulate RyR2-Ser2808 phosphorylation: local control by kinases and phosphatases in the macromolecular complex. Phosphorylation of RyR2-Ser2808 is regulated by multiple kinases and phosphatases in the RyR2 macromolecular channel complex. Anchored via leucine/isoleucine zipper motifs on the cytoplasmic NH2-terminal domain of RyR2 are PKA [via its targeting protein, myocardial A kinase-anchoring protein (mAKAP)], Ca2+-calmodulin-dependent protein kinase II (CaMKII), phosphorylate PP1 (via protein spinophilin), phosphatase P2A (via PR130), phosphodiesterase 4D3 (PDE4D3, via mAKAP), and several others, including calmodulin, FKBP12.6 (calstabin2), and sorcin (2, 18, 22, 24). Consequently, an increase in RyR2-pSer2808 could result from several events, e.g., increased PKA activity (31); downregulation of PDE4D3, which increases the local cAMP level and the PKA activity (18); or downregulation of PP1 and P2A2, which has been observed in patients with HF (24, 30, 32).

Furthermore, the RyR2-Ser2808 phosphorylation site has been proposed to be a consensus phosphorylation site not only for PKA (RRXS), but also for CaMKII (RRXS) and PKG (RR/KXS/T) (16). Western blot experiments showed that RyR2-Ser2808 could be phosphorylated, at least in vitro in SR vesicles or using purified RyR2, by PKA and, possibly, by CaMKII (33, 47) or PKG (48). Using knock-in mice in which Ser2808 has been mutated to Ala, it has been demonstrated that Ser2808 is the major, physiologically active PKA phosphorylation site on RyR2 (45). It remains to be seen whether CaMKII or PKG indeed phosphorylate RyR2-Ser2808 in vivo. Finally, it has recently been suggested that Ser2808 on RyR2 constitutes a second PKA phosphorylation site, although the physiological importance of this site remains controversial (48).

Functional consequences of RyR2-Ser2808 phosphorylation: implications in HF. The functional consequences of PKA hyperphosphorylation of RyR2 are the subject of scientific debate. Although PKA phosphorylation of RyR2 increased the single RyR2 channel activity in vitro in the planar lipid bilayers (24), the Ca2+ spark activity (each spark is generated by cooperative opening of a number of RyR2 channels in a CRU) may not be changed by PKA phosphorylation in vivo (19). Plausible explanations for this controversy include 1) incomplete PKA phosphorylation of RyR2 in isolated cardiomyocytes and 2) an increase in the opening of uncoupled RyR2 channels (“rogue RyRs”) by PKA phosphorylation of RyR2 (36), which might cause small amounts of Ca2+ leak that could not be detected by the current technique.

It has been demonstrated that, in failing hearts, RyR2 channels are more prone to abnormal SR Ca2+ release during diastole, leading to SR Ca2+ leak. One plausible explanation of enhanced SR Ca2+ release includes PKA hyperphosphorylation of RyR2-Ser2808 (44). However, in failing rabbit hearts, SR Ca2+ leak could not be reduced using the PKA inhibitor H89 but was significantly blocked by CaMKII inhibition, suggesting that abnormal CaMKII phosphorylation of RyR2 may also cause RyR2 dysregulation in HF (1). Consistent with this notion, CaMKII phosphorylation of RyR2 was shown to increase the Ca2+ spark activity in vivo (17, 13, 21), as well as the RyR2 single-channel activity in vitro (46). Given that it has been proposed that CaMKII might also phosphorylate RyR2-Ser2808 (33), the functional consequence of RyR2-Ser2808 hyperphosphorylation (alone or in combination with other phosphorylation sites on RyR2) remains to be determined.

Shortened RyR2 cluster spacing is expected to promote spontaneous Ca2+ wave generation in the failing heart. It has been proposed that derangement of Ca2+ dynamics during HF could result not only from altered RyR2 phosphorylation, but also from restructuring in the cellular organization that alters the coupling of RyR2 with the neighboring RyR2 or with other Ca2+-handling molecules (14, 37). In failing hearts of SHR, spatial dispersion of the transverse t tubule system was found to disrupt the junctional coupling between CRUs and L-type Ca2+ channels, causing asynchronous Ca2+ spark activity (37). In a previous study, we predicted that the spatial distribution of RyR2 clusters (CRUs) could also greatly affect Ca2+ wave generation (14). In the present study, we measured the distance between the RyR2 clusters and found ~10% shortening of the CRU longitudinal spacing in failing hearts of SHR. This shortening of CRU spacing might be caused by several pathological factors in failing hearts. 1) Elevated diastolic Ca2+ would cause impaired relaxation. 2) Decreased phosphorylation levels of contractile proteins, including cardiac troponin I, troponin T, and myosin light chain 2, in SHR failing hearts (20) are expected to increase myofilament sensitivity to Ca2+ and, therefore, shorten sarcomere length and RyR2 cluster longitudinal spacing (29, 38, 39). 3) Increased collagen expression in failing hearts (40) might constrain the cell length or shrink the tissue more upon processing. However, experiments using cells enzymatically digested by collagenase and protease also showed shortening of the longitudinal CRU spacing in the cells from failing hearts. Hence, the CRU spacing change in the failing heart cannot be entirely attributed to collagen and is most likely caused by the elevated diastolic Ca2+ and increased myofilament sensitivity to Ca2+.

Quantitative model simulations show that 10% shortening of CRU longitudinal spacing would greatly increase the probability of Ca2+ wave generation (14). Wave generation is further magnified when combined with an increased RyR2 activity (14), and spontaneous Ca2+ waves are known to induce abnormal electrical activity and contribute to arrhythmias (for review see Ref. 5). In support of this scenario, increased arrhythmogenic activity has been associated with the development of cardiac hypertrophy and HF in SHR (10, 27, 28).

Summary. Our longitudinal study in rats with hypertensive heart disease provides new insights into the pathogenesis of abnormal Ca2+ handling in HF. In particular, our data demonstrate that phosphorylation of RyR2-Ser2808 is increased only in SHR with end-stage HF. Moreover, our data suggest shortening of the spatial distance of CRUs in SHR failing hearts, which is expected to promote Ca2+-induced Ca2+ release from neighboring CRUs (14) and contractile dysfunction. Additional studies are required to delineate the contribution of various modifications of RyR2 and other Ca2+-handling proteins during the development of hypertensive heart disease.

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