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Differences in Sarcoplasmic Reticulum Ca^{2+} Leak Characteristics between Systolic and Diastolic Heart Failure Rabbit Models

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Abstract

Diastolic heart failure (DHF) and systolic heart failure (SHF) are two clinical subsets of chronic heart failure (CHF). Sarcoplasmic reticulum (SR) Ca^{2+} leak has been measured in SHF and might contribute to contractile dysfunction and arrhythmogenesis. However, no study has investigated a similar phenomenon in DHF. Thus, we established DHF and SHF rabbit models and compared the differences in Ca^{2+} leak between these models. New Zealand white rabbits were randomly divided into three groups (n = 8 in each group): sham operation (SO) group, DHF group and SHF group. Cardiac functions were determined by echocardiography and hemodynamic assays. The SR Ca^{2+} leak was measured with a calcium-imaging device and the expression and activities of related proteins were evaluated with Western blots and autophosphorylation. In the DHF group, there was significantly increased ventricular wall thickness and stiffness, reduced diastolic function, and total amount of FK-506 binding protein 12.6 (FKBP12.6), increased expression and activity of protein kinase A (PKA) and phosphorylation site (P2809) in the ryanodine receptor (RyR2), but no prominent Ca^{2+} leak. In the SHF group, there was significantly increased ventricular cavity size, reduced systolic function, increased SR Ca^{2+} leak, reduced total amount of FKBP12.6 and FKBP12.6-RyR2 association, increased expression and activity of PKA and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and their RyR2 phosphorylation sites with unchanged P2030. Our results suggest that a prominent SR Ca^{2+} leak was not observed in the DHF model, which may provide a new idea for the reasons in preserved systolic function, and CaMKII possibly plays a more important role in SR Ca^{2+} leak.

Key Words: systolic heart failure, diastolic heart failure, Ca^{2+} leak, ryanodine receptor, phosphorylation

Introduction

Chronic heart failure (CHF) is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood. There are two phenotypes of heart failure, systolic heart failure (SHF) and diastolic heart failure (DHF). Many studies have shown that SHF and DHF are very distinct in morphologic and functional changes although signs, symptoms and prognosis are very similar (17, 19, 38, 47). DHF refers to an abnormality of diastolic distensibility, filling or relaxation of the left ventricle with a normal left ventricular (LV) ejection fraction (EF). DHF is also referred to as heart failure with normal left ventricular ejection fraction (HFNEF) to indicate that HFNEF could be a precursor of heart failure with reduced LVEF (13, 29). Some clinical studies have indicated that DHF currently accounts for more than 50% of all heart failure patients (4).

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Despite its incidence, the mechanism of DHF is poorly understood.

Many studies have shown that abnormal release of Ca^{2+} from the sarcoplasmic reticulum (SR) *via* the cardiac ryanodine receptor (RyR2), an event called SR Ca^{2+} leak, occurs in HF and might contribute to contractile dysfunction *via* a reduction in SR Ca^{2+} content and arrhythmogenesis (7, 22). The RyR2 is the center of a large macromolecular protein complex that directly or indirectly interacts with RyR2 and modulates its function. The complex includes FK506 binding protein 12.6 (FKBP12.6), protein kinase A (PKA), Ca/CaM–dependent protein kinase (CaMKII) and others, and other associated proteins such as spinophilin, calsequestrin and sorcin. Marks and colleagues (26) have been the primary proponents of the idea that SR Ca^{2+} leak may be increased by RyR2 hyperphosphorylation for 10 years. They showed that phosphorylation by either protein PKA or CaMKII might increase RyR2 phosphorylation status and activity (26, 41, 42). RyR2 hyperphosphorylation causes FKBP12.6 dissociation from the RyR2 and increases overall open probability of RyR2 channels, which can enhance SR Ca^{2+} leak. However, the impact of PKA-mediated hyperphosphorylation and FKBP12.6 dissociation are being questioned by other groups (11, 23, 43), and all the studies were conducted in SHF patients or animal models. No study has investigated if a similar phenomenon occurs in DHF. In this study, we established a DHF and a SHF rabbit models and investigated their SR Ca^{2+} leak characteristics and potential mechanisms.

Materials and Methods

Animal Models

All animal procedures were conducted according 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). New Zealand white rabbits (2.0-2.5 kg) were randomly divided into three groups: DHF group (n = 8), SHF group (n = 8) and sham operation (SO) group (n = 8). The animals were anesthetized by intravenous injection of 3% pentobarbital sodium (30 mg/kg). The DHF model was created by surgically constricting the abdominal aorta above the renal artery by 40-50%. Specific steps are as follows. A midline incision was made, and the abdominal aorta was isolated proximal to the renal arteries. According to the abdominal aortic diameter measured by an ultrasonic diagnostic equipment, a silk ligature was tightened around both the aorta and an adjacent piece of appropriate diameter polyethylene catheter. The catheter was withdrawn immediately, producing a reduction of aortic diameter of approximately 40-50%. The SHF model was produced by creating aortic insufficiency with 4F manometer-tipped catheter (Cordis, Bridgewater, NJ, USA). Briefly, a 4F catheter connected to a pressure transducer was introduced into the left carotid artery and pushed abruptly through the aortic valve several times. Aortic insufficiency was considered adequate when the aortic pulse pressure increased by at least 50%. The SO group received sham operations (dissection of the abdominal aorta without constriction and insertion of 4F catheter without aortic destruction).

Echocardiographic Studies

Transthoracic ultrasound cardiography (UCG) studies were performed every two weeks after surgery. A UCG machine (Philips 7500, Amsterdam, Netherlands) equipped with a 7.5 MHz transducer (Philips S12, Amsterdam, Netherlands) was used to record an M-mode echocardiogram of the left ventricle and transmitral pulsed-Doppler flow velocity curves after sedation with 3% pentobarbital sodium (20 mg/kg). The M-mode recordings were manually traced to obtain anterior and posterior wall thicknesses and left ventricular inner diameters at end-diastole and end-systole. The transmitral flow velocity curves were traced to obtain peak early diastolic filling velocity (E), peak filling velocity at atrial contraction (A), their ratio (E/A), and isovolumic relaxation time (IVRT).

Hemodynamic Studies

Hemodynamic studies were performed before surgery and when symptoms of heart failure were present. A 4F catheter (Cordis, Bridgewater, NJ, USA) was introduced through the carotid artery retrograde across the aortic valve into the left ventricle. Aortic systolic blood pressure (ASBP) and aortic diastolic blood pressure (ADBP) were detected directly, and left ventricular end-diastolic pressure (LVEDP) was determined as the pressure at the point just before the onset of an increase in left ventricular systolic pressure (LVSP). The digitized left ventricular pressure recording was used to calculate the peak positive and negative values of the first derivative of left ventricular pressure (± dP/dtmax) and the time constant of the isovolumic left ventricular pressure fall (tau). After hemodynamic studies were completed, blood was sampled from the right carotid artery for serological studies, then an incision was made in the chest and the heart was quickly harvested and weighed. Apical site of the left ventricle below the papillary muscle was removed and immediately placed in liquid nitrogen.

Preparation of SR Vesicles

SR vesicles were prepared according to the method of Kranias *et al*. (21). Briefly, left ventricles were homogenized in a solution I containing 30 mM Tris-maleate, 0.3 M sucrose, 5 mg/l leupeptin, and 0.1 mM PMSF at pH 7.0. The homogenate was centrifuged at 5,500 *g* for 10 min and then at 12,000 *g* for 20 min. The pellet was suspended in solution II containing 0.6 M KCl, 30 mM Tris-maleate, 0.3 M sucrose, 5 mg/l leupeptin and 0.1 mM PMSF at pH 7.0. This suspension was centrifuged at 143,000 *g* for 45 min. The resultant pellet was suspended in solution III containing 0.1 M KCl, 20 mM Tris-maleate, 0.3 M sucrose, 5 mg/l leupeptin, and 0.1 mM PMSF at pH 7.0. This suspension represented the microsomal fraction that was rich in SR vesicles.

Ca^{2+} Uptake and Leak Assays

Ca^{2+} uptake and Ca^{2+} leak were measured as described by Doi *et al*. (6). SR vesicles (0.2 mg/ml) were incubated in 0.5 ml of a solution containing 0.15 mM potassium gluconate, 1 mM $MgCl_2$, 0.2 mM EGTA-calcium buffer (free [Ca^{2+}], 0.3 μM), 10 mM NaN_3, and 20 mM MOPS at pH 6.8. Ca^{2+} uptake was initiated by the addition of 0.5 mM ATP at 37°C. After the Ca^{2+} uptake had reached a plateau, 1 μM thapsigargin (Sigma, St. Louis, MO, USA) was added to inhibit SR Ca^{2+}-ATPase activity with or without FK506 (30 μM, Sigma) and monitored the resultant Ca^{2+} leak. The time course of Ca^{2+} uptake and Ca^{2+} leak was monitored by a cooled CCD camera (Coolsnap ES, Photometrics, Tucson, AZ, USA) using fura-2 as a Ca^{2+} indicator (excitations at 340 and 380 nm, emission at 490 nm), image and data were analyzed by MetaFluor software (Molecular Devices, Downingtown, PA, USA). The magnitude of the Ca^{2+} leak was taken as the value obtained 60 seconds after the addition of thapsigargin, and it was expressed as a percentage of the preceding Ca^{2+} uptake.

Coimmunoprecipitation and Immunoblot Analysis

LV tissue homogenates were immunoprecipitated with an anti-RyR2 antibody, and samples were size-fractionated with SDS-PAGE for transfer onto nitrocellulose membranes. Membranes were probed with primary antibodies anti-PKA (at 1:1000 dilution), anti-CaMKII (1:1000), anti-RyR2 (1:2000) (Abcam, Leeds, UK) and anti-FKBP12/12.6 (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated RyR2 (P2815, P2030 and P2809) (1:4000) (Badrilla, Leeds, UK), and glyceraldehyde phosphate dehydrogenase (GAPDH) (1:200) (Millipore, Billerica, MA, USA). After washing, membranes were incubated with secondary antibodies (IgG) (KPL, Gaithersburg, MD, USA) conjugated to horseradish peroxidase (1:1000 dilution). Furthermore, LV tissue homogenates were subjected to Western blot analysis to measure the total expression amount of FKBP12.6. Protein-antibody reactions were detected by chemiluminescence using Kodak X-Omat films. The relative amounts of the proteins on the blots were determined by densitometric analysis using a HP laser scanner and Quality One software (BIO-RAD, Hercules, CA, USA). Protein expression level was normalized to that of GAPDH.

PKA and CaMKII Activity Assay

The activities of PKA and CaMKII were measured with the cAMP-Dependent Protein Kinase (PKA) Assay System and Calcium/Calmodulin-Dependent Protein Kinase (CaMKII) Assay System, respectively (Promega, Madison, WI, USA) (10). The SR vesicles were centrifuged at 14,000 g for 5 min, and the supernatant was saved as the enzyme sample. The samples were mixed with the reaction solution containing PKA (or CaMKII) 5× assay buffer, 0.025 mM cAMP, PKA (or CaMKII) biotinylated peptide substrate, [γ-^{32}P] ATP mix, and 0.1 mg/ml BSA at 30°C for 5 min. After terminating the reaction, 10 μl of each reaction was spotted onto a prenumbered SAM membrane square following the wash and dry steps. Scintillation fluid was added to the vials before counting.

Statistical Analysis

Data are expressed as means ± standard deviation (SD). Intergroup analysis was performed by one-way analysis of variance (ANOVA) with a *post hoc* Schiff's test. Statistical significance was taken at a value of $P < 0.05$.

Results

UCG and Doppler Examination

In the DHF group, left ventricular relative wall thickness progressively increased to 12 weeks, and left ventricular end-systolic internal diameter (LVIDs) and end-diastolic internal diameter (LVIDd) were not different at 12 weeks between DHF and SO rabbits (Table 1, Fig. 1). Moreover, the early diastolic filling wave and E/A ratio were significantly increased from 8 to 12 weeks after surgery, and accompanied with prolongation of the isovolumic relaxation time (IVRT) without an altered EF and mid-wall fractional shortening (FS). There were significant differences between the DHF group and the other two groups ($P < 0.05$

Table 1. Comparison of UCG parameters in three rabbit models

	SO group (n = 8)		DHF group (n = 8)		SHF group (n = 8)	
	Pre-operation	Post-operation 12 weeks	Pre-operation	Post-operation 12 ± 2 weeks	Pre-operation	Post-operation 12 ± 1 weeks
IVSd (mm)	1.98 ± 0.33	2.21 ± 0.35	2.0 ± 0.16	3.64 ± 0.51**##	1.93 ± 0.21	2.35 ± 0.36
LVPWd (mm)	1.95 ± 0.31	2.12 ± 0.33	2.05 ± 0.28	3.53 ± 0.48**##	1.91 ± 0.26	2.14 ± 0.4
LVIDd (mm)	14.4 ± 1.3	14.8 ± 1.9	15.3 ± 1.7	13.7 ± 1.9##	15.1 ± 1.2	18.4 ± 2.1**
LVIDs (mm)	9.11 ± 1.12	9.34 ± 1.24	9.31 ± 1.26	8.71 ± 1.88##	9.45 ± 1.38	14.9 ± 2.17**
FS (%)	38.6 ± 6.6	40 ± 4.9	37.4 ± 6.3	41.5 ± 6.7##	39.11 ± 4.5	24.7 ± 5.9**
EF (%)	69.3 ± 5.3	70.3 ± 8.1	70.6 ± 6.2	73.4 ± 6.8##	70.9 ± 5.5	41 ± 8.5**
E/A	1.27 ± 0.2	1.46 ± 0.22	1.23 ± 0.19	2.41 ± 0.42**##	1.26 ± 0.17	1.39 ± 0.35
IVRT (ms)	39.5 ± 8.69	42.63 ± 8.16	40.88 ± 8.11	58.75 ± 13.42**##	42.88 ± 6.75	45.38 ± 6.19

Data are shown as means ± SD. IVSd, interventricle septal diameter; LVPWd, left ventricular posterior wall diameter; LVIDd, left ventricular internal diameter diastolic; LVIDs, left ventricular internal diameter systolic; FS, fraction shortening; EF, ejection fraction; E/A, the ratio of peak early diastolic filling velocity and peak filling velocity at atrial contraction; IVRT, isovolumic relaxation time.

*$P < 0.05$, **$P < 0.01$ compared with SO group.

#$P < 0.05$, ##$P < 0.01$ DHF group compared with SHF group.

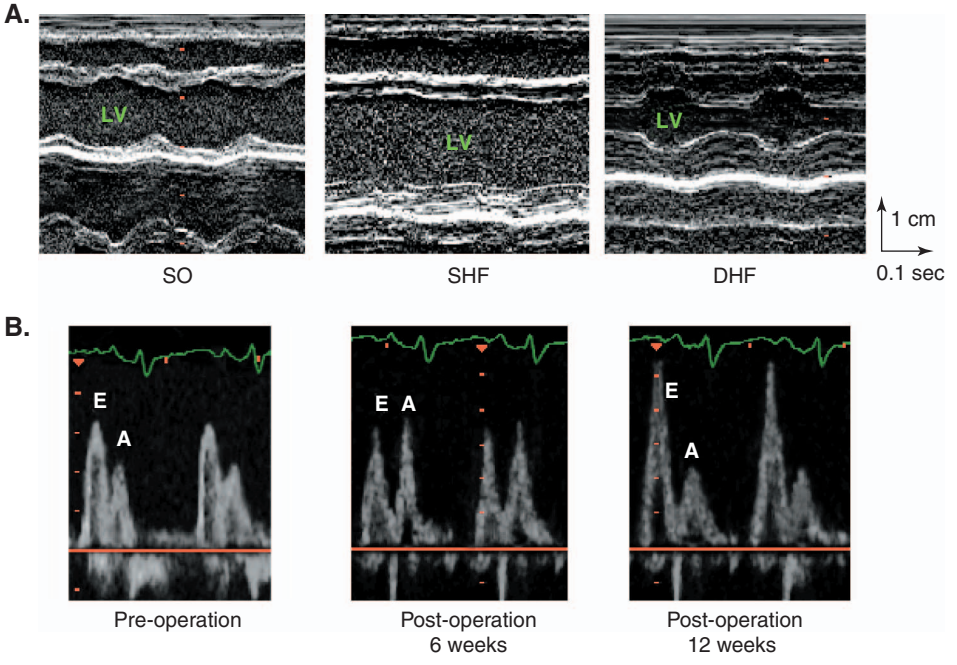

Fig. 1. A. Representative M-mode echocardiogram. Note that LVIDd and LVIDs were bigger in the SHF rabbits, and smaller in the DHF rabbits. B. Evolving mitral flow velocity patterns in DHF model. A = peak filling velocity at atrial contraction; E = peak early diastolic filling velocity. Note that peak early diastolic filling velocity and E/A ratio slightly and progressively decreased in post-operation 2-6 weeks and were almost constant at 6-7 weeks, followed by an abrupt increase at 12 ± 2 weeks.

or $P < 0.01$). In the SHF group, LVIDd and LVIDs progressively increased to 12 weeks when heart failure occurred with a significant reduction of the EF and FS values. The relative wall thickness of the left ventricular was not different at 12 weeks, and the diastolic function was also unchanged during the observation stage. There were significant differences between the SHF and the other groups ($P < 0.05$ or $P < 0.01$).

Hemodynamic Studies

After surgery, ASBP and ADBP were increased in the DHF group (Table 2). When symptoms of heart

Table 2. Comparison of hemodynamic, weight index and serological index in the three models at 12 weeks post-operation

	SO group (n = 8)	DHF group (n = 8)	SHF group (n = 8)
HR (min^{-1})	233 ± 27	273 ± 36*	283 ± 46*
ASBP (mmHg)	111 ± 20	176 ± 29**##	132 ± 26
ADBP (mmHg)	82 ± 6	98 ± 13**##	86 ± 7
LVEDP (mmHg)	-5 ± 3	19 ± 7##	18 ± 5**
LV+dp/dt_{max} (mmHg/s)	4,655 ± 772	4,706 ± 792##	3,521 ± 646**
LV-dp/dt_{max} (mmHg/s)	3,760 ± 685	2,892 ± 565**##	3,608 ± 629
tau (ms)	17 ± 3	26 ± 5**##	19 ± 4
LV mass index (mg/g)	1.41 ± 0.6	2.94 ± 0.81**	3.11 ± 0.93**
LW/BW (mg/g)	3.68 ± 0.87	5.98 ± 0.91**##	6.77 ± 1.24**
BNP (pmol/ml)	8.72 ± 2.37	34.55 ± 7.88**	33.61 ± 6.26**
NE (pg/ml)	81.83 ± 17.6	189.93 ± 29.07**	192.03 ± 38.02**
EPI (pg/ml)	6.32 ± 2.34	24.61 ± 6.41**	23.84 ± 5.17**

Data are shown as means ± SD. HR, heart rate; ASBP, aortic systolic blood pressure; ADBP, aortic diastolic blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; LV dp/dt_{max}, first derivatives of left ventricular value of systolic and end-diastolic pressure; tau, time constant of isovolumic left ventricular pressure fall; LV mass index, left ventricular mass index; LW/BW, the ratio of lung weigh and body weigh; BNP, B-type brain natriuretic peptide; NE, norepinephrine; EPI, epinephrine.
*$P < 0.05$, **$P < 0.01$ compared with SO group.
#$P < 0.05$, ##$P < 0.01$ DHF group compared with SHF group.

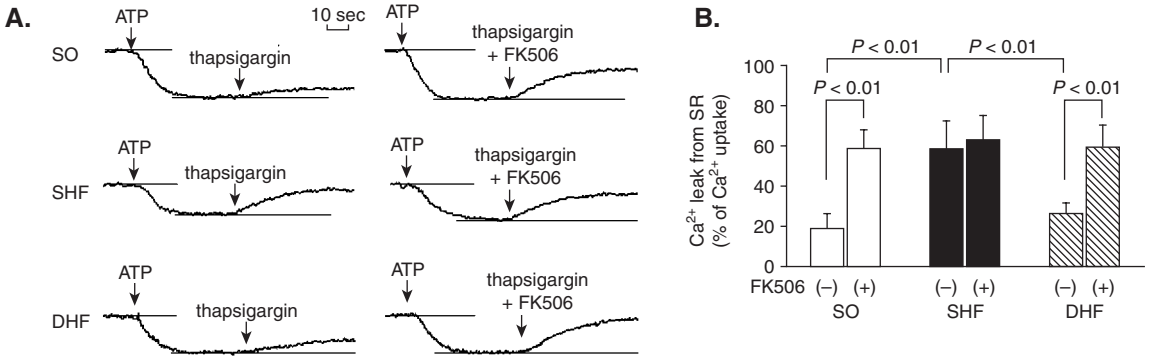

Fig. 2. A. Representative time courses of Ca^{2+} uptake and the ensuing Ca^{2+} leak from SR vesicles obtained from the SO, SHF and DHF hearts. Note that the spontaneous Ca^{2+} leak had been seen in SHF SR vesicles. Note also that FK506 enhanced the Ca^{2+} leak in the SO and the DHF rabbits but not in the SHF rabbits. B. Comparison of spontaneous and FK506-induced Ca^{2+} leaks in SR vesicles from the SO, SHF and DHF groups (n = 8).

failure were present, the LVEDP was significantly increased in both the DHF and SHF groups as compared to the SO group ($P < 0.01$). Moreover, there was a significant decrease of -dP/dt_{max} and an increase of tau in the DHF group at 12 weeks after surgery as compared with those of the SO and SHF group ($P < 0.01$). The LV mass index (LV weight/body weight) and lung weight/body weight were significantly increased in both the DHF and SHF groups ($P < 0.01$). Furthermore, BNP (B-type brain natriuretic peptide), NE (norepinephrine) and EPI (epinephrine) were significantly increased in the sera of the DHF and SHF groups ($P < 0.01$).

Ca^{2+} Uptake and Leak Assays

Addition of thapsigargin to normal SR vesicles produced a small Ca^{2+} leak, whereas addition of FK506 together with thapsigargin produced a much more pronounced leak (Fig. 2). In contrast, in the SHF group, addition of thapsigargin alone produced a prominent SR Ca^{2+} leak; however, addition of FK506 produced no additional increase. In the SR vesicles from DHF rabbits, an obvious spontaneous Ca^{2+} leak was not observed after addition of thapsigargin, and FK506 had the same effect as in normal SR.

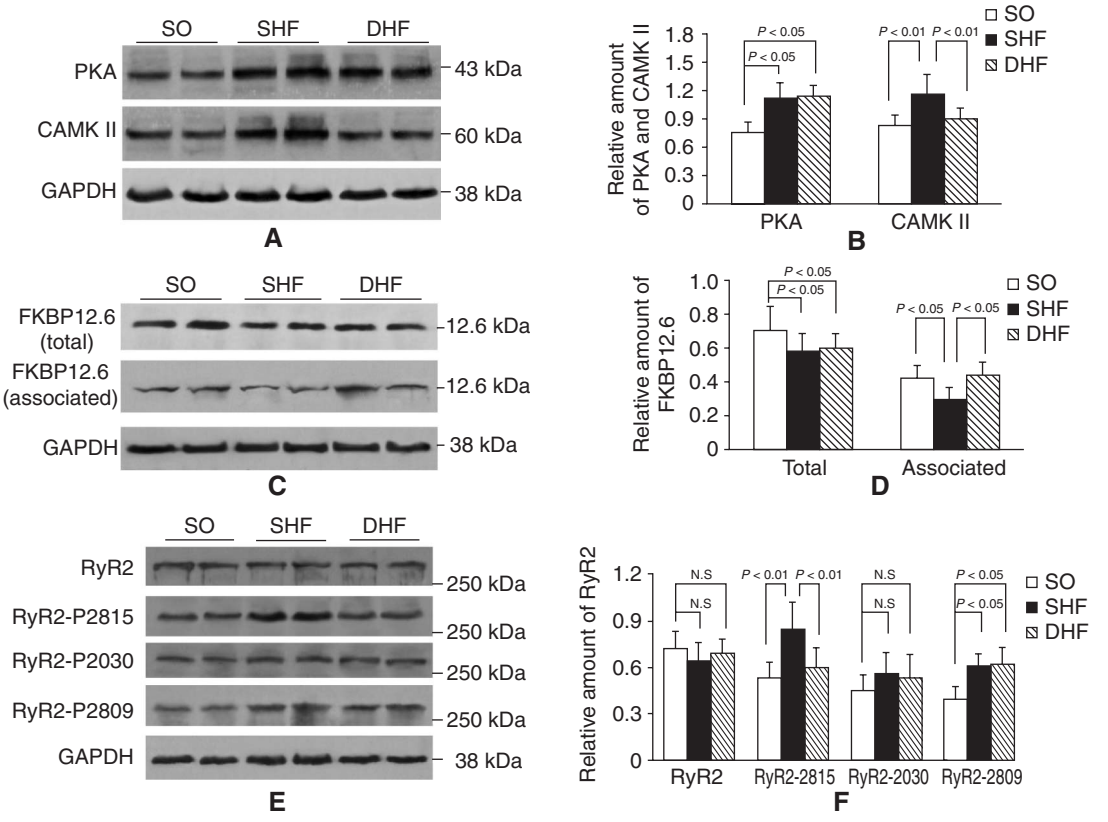

Fig. 3. Relative amounts of related proteins. (A) Western blots of PKA, CaMKII and GAPDH from immunoprecipitated LV tissue homogenates with RyR2 in every group and pooled data (B) including ratios of PKA and CaMKII to GAPDH (n = 8). (C) Immunoblots of FKBP12.6 and GAPDH from immunoprecipitated and unimmunoprecipitated LV tissue homogenates with RyR2 in every group and pooled data (D) including ratio of FKBP12.6 to GAPDH (n = 8). (E) Western blots of RyR2, RyR2-P2815, RyR2-P2030, RyR2-P2809 and GAPDH from immunoprecipitated LV tissue homogenates with RyR2 in every group and pooled data (F) including ratios of RyR2, RyR2-P2815, RyR2-P2030 and RyR2-P2809 to GAPDH (n = 8).

PKA and CaMKII Expression and Activation

Alterations of PKA and CaMKII expression and activation state could mediate PKA or CaMKII-dependent phosphorylation of RyR2 in HF. So we respectively measured PKA and CaMKII expression on local (associated with RyR2) levels using Western blots and activity in the SR vesicles using autophosphorylation. PKA and CaMKII protein expression in the three groups is shown in Fig. 3, A and B. Significant upregulation of PKA expression was found in both the SHF and DHF groups ($P < 0.05$), but increased CaMKII expression was only noted in the SHF group ($P < 0.01$). Moreover, PKA and CaMKII activities were significantly increased in the SR vesicles from SHF ($P < 0.01$), whereas there was increased PKA and unchanged CaMKII activity in the SR vesicles from DHF ($P < 0.01$). A comparison of the activities of PKA and CaMKII in the three groups is presented in Fig. 4.

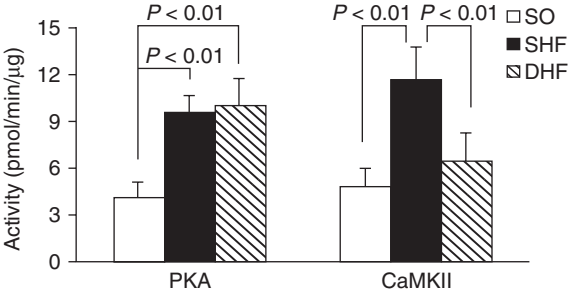

Fig. 4. Comparison of the activities of PKA and CaMKII in the SO, SHF and DHF groups (n = 8).

FKBP12/12.6 Expression and Association with RyR2

We measured the total amount of FKBP12.6 in LV tissues by Western blots. The results showed the total amount of FKBP12.6 was lower in both the SHF and DHF groups than in the SO group (Fig. 3, C and D), there were significant differences between the two HF groups and the SO group ($P < 0.05$). Altered RyR2 phosphorylation could influence FKBP12.6-RyR2 association; therefore, we assessed how much FKBP12.6

was associated with immunoprecipitated RyR2. The level of coimmunoprecipitated FKBP12.6 significantly decreased only in the SHF group and there were significant differences between the SHF group and the other two groups ($P < 0.05$).

RyR2 Expression and Phosphorylation Status

RyR2 protein expression was assessed by Western blot. In LV homogenates from the SHF hearts, there was a reduced trend in RyR2 protein expression, but the difference with the other groups did not reach statistical significance (Fig. 3, E and F). RyR2 contains multiple phosphorylation sites including RyR2-Ser2815 (phosphorylated by CaMKII), RyR2-Ser2809 (phosphorylated by both CaMKII and PKA) (32) and RyR2-Ser2030 (phosphorylated only by PKA) (42). We measured the RyR2 phosphorylation status by using the three important phosphospecific antibodies and found that RyR2 phosphorylation was significantly increased at P2809 in both the SHF and DHF groups ($P < 0.05$), at P2815 only in the SHF group ($P < 0.01$), whereas there was a increased trend at P2030 in two HF groups, but the difference with the SO group did not reach statistical significance ($P > 0.05$).

Discussion

CHF is a complex syndrome which includes disturbed ventricular function, neurohumoral and cytokine activation and increased arrhythmias. The most frequent causes of HF are myocardial infarction, pressure overload, volume overload, viral myocarditis and toxic cardiomyopathy. Our heart failure models were established by pressure overload and volume overload. The diagnostic criteria of CHF are: 1) signs or symptoms of CHF; 2) normal (in DHF) or abnormal systolic LV function (in SHF) and evidence of diastolic LV dysfunction (as determined by UCG and hemodynamic parameters in DHF); and 3) increased LVEDP and BNP. In the SHF animals in this study, there was a significant enlargement of the left ventricular chamber, decreased EF and increased LVEDP. In the DHF animals, the ventricular wall thickness and stiffness, E/A and LVEDP were significantly increased and IVRT and tau were prolonged without a change in EF. These features in our animal models are in agreement with the clinical phenotypes of the two types of HF reported in many studies (2, 3, 20, 37, 39) indicating that the HF models in the current study were successfully established.

Kawai and Konishi (18) reported that a small amount of Ca^{2+} leak from cardiac SR into the cytoplasm during the resting state, even under physiological conditions. Many recent studies have shown that the Ca^{2+} leak becomes more prominent in the failing heart leading to depression of contractile function or arrhythmia (9, 24, 34, 36). No studies this far have reported a similar phenomenon in DHF models. This study is the first to show SR Ca^{2+} leak in DHF SR vesicles. After the initiation of Ca^{2+} uptake, thapsigargin, an inhibitor of SR Ca^{2+}-ATPase activity, was added to SR vesicles in each group. Ca^{2+} release from RyR2 thereafter was defined as spontaneous Ca^{2+} leak. Furthermore, FK506, which binds to the FKBP12.6 associated with the RyR2, could cause an induced Ca^{2+} leak even in normal SR vesicles (28, 45). In this study, a prominent spontaneous Ca^{2+} leak was observed even in the absence of FK506, and FK506 produced little or no further increase in Ca^{2+} leak in SHF SR vesicles. Only a small spontaneous Ca^{2+} leak was observed without FK506, and FK506 produced a significant increase in Ca^{2+} leak in both the SO and DHF SR vesicles. However, currently we can not interpret the differences in Ca^{2+} leak between the two HF models, which may be the result of an alternative response to the initial stimuli or a different type of heart failure between them. Further research to verify this possibility is required.

The major causes of SR Ca^{2+} leak are reduced amount of FKBP12.6 (14, 30, 40) and RyR2 hyperphosphorylation by PKA and/or CaMKII leading to FKBP12.6 dissociation, increased RyR Ca^{2+} sensitivity and higher RyR channel Ca^{2+} flux (1, 12, 25, 27). However, the functional significance of RyR phosphorylation by PKA or CaMKII remains unclear in SR Ca^{2+} leak in HF (12, 23, 26, 42, 43). Therefore, we chose all phosphorylation sites to detect their respective effects on SR Ca^{2+} leak. As we expected, there was reduced total and associated amount of FKBP12.6, increased expression and activity of PKA and CaMKII, and increased RyR2 phosphorylation (both P2815 and P2809) in the SHF group. And there was increased expression and activity of PKA and its RyR2 phosphorylation site P2809 with reduced total amount of FKBP12.6 and unchanged CaMKII expression and its phosphorylation site P2815 in the DHF group. Furthermore, there was no significant difference in total RyR2 protein expression and phosphorylation site P2030 among three groups. The above-mentioned results further verify differences in SR Ca^{2+} leak between SHF and DHF groups.

FKBP12.6 has been proposed to stabilize the closed state of the RyR2 channel and its dissociation has been shown to enhance SR Ca^{2+} leak (30, 33, 40, 46). However, a number of recent studies have shown that FKBP12.6 removal has little or no effect on RyR2 activity (8, 11, 44) and have questioned its role in HF. Therefore, as both the dissociation and result of FKBP12.6 are controversial, it is important to determine and compare the role of FKBP12.6 in SHF

and DHF animal models. In this study, the total amount of FKBP12.6 was reduced in both the HF groups, but the amount of FKBP12.6 associated with RyR2 was reduced only in the SHF group. Because the associated amount of FKBP12.6 played more important role in SR Ca^{2+} leak, enough FKBP12.6 associated with RyR2 in the DHF group enhanced channel stabilization and reduced Ca^{2+} leak. As for the difference of FKBP12.6-RyR2 association could result from changed RyR2 phosphorylation status.

PKA and CaMKII are two key kinases involved in the regulation of calcium cycling by phosphorylating calcium handling proteins such as phospholamban (PLB) and RyR2. Some early studies showed that PKA-mediated hyperphosphorylation of RyR2 at S2809 in the failing heart caused increased RyR2 Ca^{2+} sensitivity and abnormal channel activity by the dissociation of FKBP12.6 from the RyR2. There is also compelling evidence from the Bers laboratories that CaMKII mediated RyR2 phosphorylation at Ser-2815 is responsible for SR Ca leak (1, 5, 23). However, it is controversial about which kinase, PKA or CaMKII, plays more key role in the SR Ca^{2+} leak in HF. Li *et al*. found that PKA-dependent RyR2 phosphorylation did not alter resting RyR2 function in mouse myocytes (23). It was also reported that PKA phosphorylation at RyR2-Ser2808 did not dissociate FKBP12.6 from the RyR2 (11, 43). There are now much more data refuting than supporting the hypothesis that PKA-mediated RyR-S2809 phosphorylation is a critical regulator of RyR2 function in SR Ca^{2+} leak. Therefore, CaMKII hyperphosphorylation-mediated Ca^{2+} leak will be a new focus of the research in the future. Furthermore, the third identified site in the RyR2 is S2030 which was described as being phosphorylated only by PKA. Many studies have indicated that S2030 is a major PKA phosphorylation site in RyR2 responding to acute β-adrenergic stimulation, but it is not hyperphosphorylated by PKA in HF models (15, 16), which is in agreement with our study. Hence, its role on SR Ca^{2+} leak needs to be further investigate. Our results also showed that increased PKA expression and activity might enhance phosphorylation of RyR2-P2809 in both SHF and DHF, whereas increased CaMKII expression and activity might enhance phosphorylation of RyR2-P2815 only in SHF. However, we only found a prominent SR Ca^{2+} leak in the SHF group, which is possibly because PKA-dependent RyR2 phosphorylation alone can not enhance SR Ca^{2+} leak. CaMKII may be a more key modulator in SR Ca^{2+} leak and can enhance SR Ca^{2+} leak alone or in combination with PKA by phosphorylating RyR2 (1, 23, 25, 35, 43). As for the difference of CaMKII expression between the DHF and SHF groups, the major reason may be differences of initial stimulus and degree of neurohumoral activation between the two types of HF.

In conclusion, we found that a prominent Ca^{2+} leak through RyR2 was present in the rabbit model of SHF, but only a small Ca^{2+} leak was observed in the DHF model. The expression and phosphorylation status of related proteins also support the results, which may provide a new idea for the reasons of preserved systolic function in DHF. Furthermore, our results also support that CaMKII possibly plays a more important role in SR Ca^{2+} leak and can affect RyR2 alone or in association with PKA.

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