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Assessment of sarcoplasmic reticulum Ca^{2+}-uptake during the development of left ventricular hypertrophy

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Abstract

Cardiac sarcoplasmic reticulum (SR) sequesters Ca^{2+} and plays a crucial role in the regulation of intracellular Ca^{2+}. Its functional properties are central to the excitation–contraction cycle of cardiac muscle. In this study, we hypothesized that alterations in SR function occur during the development of left ventricular (LV) hypertrophy. LV hypertrophy was produced in Lewis rats by the one-kidney, one-clip (1K1C) procedure. LV tissues were obtained from 1K1C rats 1 week (mild, N = 7), 4 weeks (moderate, N = 7), and 8 weeks (severe, N = 7) post-surgery and from the corresponding age-matched, sham-operated controls (N = 7 at each stage). In all of these rats, the ratio of LV weight (g) to body weight (kg) was determined and considered as an index for LV hypertrophy. In addition, the ratio of lung weight (g) to body weight (kg) was determined and considered as an index for pulmonary congestion and heart failure. In each LV specimen, SR Ca^{2+}-uptake and tissue Ca^{2+}-ATPase (CAA) level were determined. In 1K1C rats, LV hypertrophy increased by 21, 40, and 90% at 1, 4, and 8 weeks post-surgery, respectively, compared to the age-matched, sham-operated rats, whereas pulmonary congestion did not occur at 1 and 4 weeks but increased significantly by about 21% at 8 weeks. Further, both SR Ca^{2+}-uptake and immunodetectable CAA level did not change at 1 week, increased (54%) to the same extent at 4 weeks, and decreased (42%) by approximately the same extent at 8 weeks in 1K1C rats compared to the age-matched, sham-operated rats. In summary, as LV hypertrophy evolved, Ca^{2+}-uptake and CAA expression did not change in the early, increased in the moderate, and then declined in the later stages of hypertrophy development. The increase in Ca^{2+}-uptake and CAA expression suggests, at the cellular level, a compensatory response to LV hypertrophy, while the decline at later stages indicates the transition to heart failure.

Keywords: LV hypertrophy; Sarcoplasmic reticulum; Ca^{2+}-uptake; Ca^{2+}-ATPase

1. Introduction

LV hypertrophy is a hallmark characteristic of heart failure. In response to a variety of physiological and pathological stimuli, LV myocardium becomes hypertrophied to adapt to an increase in workload [1]. The most common stimulus is pressure-overload resulting from systemic hypertension. If the increase in LV pressure persists, alterations in the properties of the chamber muscle evolve into congestive heart failure [2,3]. Earlier investigations have suggested that the changes in excitation–contraction coupling, in particular altered SR Ca^{2+} handling, could be responsible for the contractile defects in hypertrophied and failing hearts [4]. In normal hearts, the SR serves as a sink for Ca^{2+} ions during relaxation and as a Ca^{2+} source during contraction. The important proteins in the SR membrane for Ca^{2+}-uptake are CAA and PLB [5,6]. In addition, the SR also contains CSQ for Ca^{2+} storage within SR vesicles and protein kinases (cyclic AMP-dependent and Ca^{2+}-calmodulin-dependent protein kinases) to regulate SR Ca^{2+}-uptake by modulating the phosphorylation states of PLB and CAA [5–7].

Several investigators have observed reduced SR Ca^{2+}-uptake in failed [8,9] and severely hypertrophied hearts [10]. Using different models of LV hypertrophy, a number of studies have attempted to distinguish the biochemical changes pertaining to SR function during mild or moderate

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Abbreviations: SR, sarcoplasmic reticulum; LV, left ventricle (left ventricular); MV, membrane vesicles; 1K1C, one-kidney, one-clip; CAA, Ca^{2+}-ATPase; PLB, phospholamban; and CSQ, calsequestrin.

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(compensated) and severe (decompensated) LV hypertrophy. Limited data are available that demonstrate any improvement in SR function during compensated LV hypertrophy [11,12]. Several investigators studied SR function in LV hypertrophy using different models, such as aortic-banded hypertrophied hearts [11–18], left coronary artery ligated hearts [19–23], and volume-overloaded hypertrophied hearts [24,25]; however, only a few of them [11,12] have reported biphasic changes in SR Ca^{2+}-uptake during the development of LV hypertrophy. SR function during the development of LV hypertrophy has not been assessed in the 1K1C rat model, whereby LV hypertrophy develops due to pressure-overload. In the present study, we evaluated LV hypertrophy, pulmonary congestion, and LV SR Ca^{2+}-uptake and CAA protein expression level in 1K1C Lewis rats at 1, 4, and 8 weeks post-surgery and in age-matched, sham-operated controls.

2. Materials and methods

2.1. Materials

Antibody specific to CAA and CSQ was obtained from Affinity Bioreagents, Inc. Chemicals and supplies for electrophoresis and electrotransfers were purchased from Bio-Rad. General chemical supplies used in the preparation of MV, LV homogenate, and the measurement of SR Ca^{2+}-uptake were obtained from the Sigma Chemical Co.

2.2. Preparation of animals

Ventricular hypertrophy was produced in rats using the Goldblatt's 1K1C rat model of systemic hypertension [26]. Lewis rats, weighing 180–220 g, were anesthetized with pentobarbital (30–35 mg/kg, i.p.). Under sterile conditions, the abdominal cavity was opened via a middle incision and the left and right renal artery and veins were exposed. One kidney, selected at random, was removed after ligation of the renal artery and vein. An implantable metal clip was placed on the renal artery of the second kidney to produce a 70–75% lumenal stenosis. The abdominal cavity was then closed with 3-O silk sutures, and the rats were allowed to recover. Rats were divided into three study groups: one group were killed 1 week after the 1K1C surgical procedure, the second group at 4 weeks, and the third group at 8 weeks. Age-matched, sham-operated control animals also underwent the same operation, but a kidney was not removed and a metal clip was not placed on the renal artery of the second kidney. Just before being killed, the body weight of the animal was taken in kilograms. At the time of killing, while under general anesthesia, the chest was opened, and the heart with lungs was rapidly removed and placed in ice-cold saline solution. The LV and lungs were separated, weighed in grams, quickly frozen in liquid nitrogen, pulverized, and stored at -70° until used. LV hypertrophy was determined by calculating the ratio of LV weight (g) to body weight (kg), and the pulmonary congestion was determined by calculating the ratio of lung weight (g) to body weight (kg).

2.3. Preparation of MV

Approximately 250 mg of LV powder from each sham or 1K1C rat was homogenized individually in 10 vol. of ice-cold medium-1 (10 mM Tris-maleate, pH 7.0, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, 0.3 M sucrose, 1 mM sodium pyrophosphate, 1 mM benzamidine, 0.5 μg/mL of aprotinin, and 0.5 μg pepstatin). EDTA, EGTA, and sodium pyrophosphate were added during homogenization to maintain the state of phosphorylation of proteins by inhibiting protein kinase and phosphatase activities, whereas protease inhibitors were added to protect SR proteins from proteolysis during the isolation procedure. Homogenization for 3×20 min was carried out by using a 7-mm generator (Omnimixer, Inc.) set at maximal speed. The homogenate was filtered through four layers of cheesecloth and then centrifuged for 30 min at 100,000 *g* at 4°. The resulting pellet was washed twice with 10 mL of medium-1 containing 0.6 M KCl and once with 10 mL of medium-2 (10 mM Tris-maleate, pH 7.0, 0.3 M sucrose) by resuspending the pellet and then recentrifuging at 100,000 *g* for 30 min at 4°. The final resulting pellet, consisting of almost all of the longitudinal and junctional SR membrane vesicles present in the starting sample (referred to as MV), was resuspended in 1 mL of medium-2. The suspension was aliquoted into 200 μL/tube and then rapidly frozen in liquid nitrogen and stored at -70° for subsequent analysis. The protein concentration of the MV was measured as previously described [27] with bovine serum albumin used as a standard.

2.4. SR Ca^{2+}-uptake

Oxalate-dependent Ca^{2+}-uptake, a characteristic of the SR, was determined in the MV at various Ca^{2+} concentrations (0.1 nM to 10 μM) according to a method used previously by us in dogs [28]. The Ca^{2+}-uptake assay contained 20 μM ruthenium red to inhibit Ca^{2+} release from the SR. In addition, 1 mM lanthanum chloride was included in the washing buffer to block the release of Ca^{2+} from the SR during washing. Ca^{2+}-uptake was expressed as nanomoles $^{45}Ca^{2+}$ accumulated per minute per milligram protein. A computer program was used to calculate free Ca^{2+}concentrations for Ca^{2+}-uptake [28]. Kinetic parameters, maximal velocity (V_{max}), and the affinity of Ca^{2+}-uptake for Ca^{2+} ($K_{0.05}$) were calculated as described previously [28].

2.5. Western blotting

The protein level of CAA was determined in the SDS-extract of MV isolated from the LV tissue of sham-operated

and 1K1C rats. In addition, the protein level of CSQ, which was reported to be unchanged during the development of LV hypertrophy or heart failure [13,15,23], was also determined as an internal control for protein loading. Western blotting was performed using a modification of the method described previously [28,29]. Approximately 1–100 μg of the SDS-homogenate extract was subjected to 4–20% linear polyacrylamide gel electrophoresis (Bio-Rad). Subsequently, the separated proteins were transferred electrophoretically onto a nitrocellulose membrane. The accuracy of the electrotransfer was confirmed by staining the membrane with 0.1% amido black. The membrane blot was then incubated with primary antibody as previously described [28,29]. Monoclonal antibody was diluted 1000-fold for CAA and 500-fold for CSQ. Primary-antibody binding proteins were detected by incubating the blot with a peroxidase-conjugated anti-mouse secondary antibody (Sigma) and visualized using enhanced chemiluminescence (Dupont-NEN). The intensity of the bands was quantified using a Bio-Rad model GS-670 imaging densitometer. The densitometric unit of measurement was OD × mm^2. When the autoradiogram was exposed for just 10 sec, a linear correlation was observed between densitometric units and protein content up to 100 μg protein for both CAA and CSQ, as shown in Fig. 1. Bands on the same autoradiograph were very prominent when the film was exposed for more than 30 sec (data not shown). In another experiment where we carried out a similar experiment with 1–20 μg of the SDS-extract, as defined above for 1–100 μg SDS-extract protein, and exposed the film for 1 min, a linear correlation was observed between the densitometric units and the protein content from 1 to 20 μg protein for both CAA and CSQ (data not shown).

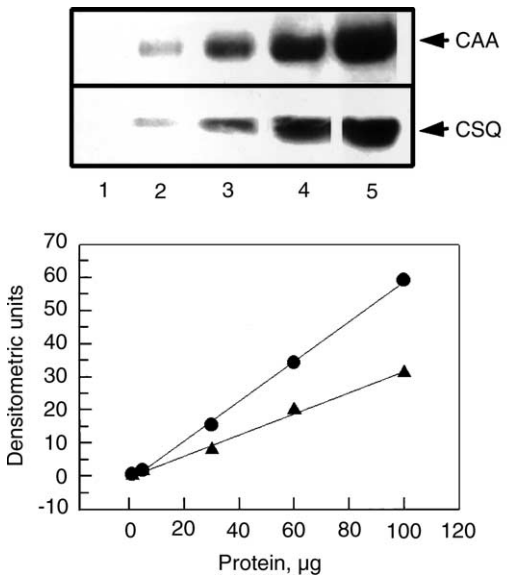

Fig. 1. Western blot (top) and densitometric analysis of the western blot (bottom) showing dependency of immunodetectable sarcoplasmic reticulum CAA and CSQ in an SDS-extract of LV myocardium of normal dogs. Immunodetection is shown with increasing protein concentration in the range of 1–100 μg. This figure is representative of three experiments.

2.6. Statistical analysis

Data are shown as means ± SEM. Statistical comparisons among the groups were performed using one-way ANOVA with alpha set at 0.05. When significance was obtained, pair-wise comparisons were performed using the Student–Neuman–Keuls test. For this test, a probability of $P < 0.05$ was considered significant.

3. Results

3.1. LV hypertrophy and pulmonary congestion

The increase in the ratio of LV weight (LVW) to body weight (BW) was regarded as an index for LV hypertrophy. As shown in Fig. 2, the LVW/BW ratio in 1K1C rats significantly increased as early as 1 week after surgery (2.45 ± 0.07 vs 2.02 ± 0.02, $N = 7$) and continued to increase further at 4 weeks (2.81 ± 0.04 vs 2.01 ± 0.03, $N = 7$) and 8 weeks (3.84 ± 0.08 vs 2.02 ± 0.02, $N = 7$), compared to the age-matched, sham-operated controls. There was no difference in the LVW/BW ratio among 1-, 4-, and 8-week sham-operated control rats. The increase in the ratio of lung weight (LW) to body weight (BW) was regarded as an index for pulmonary congestion and LV failure. The LW/BW ratio did not change in 1K1C rats at 1 or 4 weeks post-surgery but increased about 21% at 8 weeks post-surgery (7.03 ± 0.15 vs 5.84 ± 0.09, $N = 7$) compared to the age-matched, sham-operated controls (Fig. 3). No changes in this ratio were observed among 1-, 4-, or 8-week sham-operated control rats.

3.2. SR Ca^{2+}-uptake

The maximal velocity (V_{max}, nmol $^{45}Ca^{2+}$ accumulated into the SR/min/mg protein) of oxalate-dependent SR

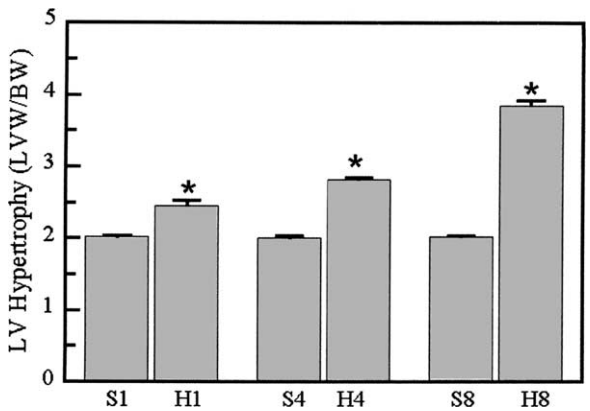

Fig. 2. Ratio of left ventricular weight (LVW) to body weight (BW) in 1-week (S1, H1), 4-week (S4, H4), and 8-week (S8, H8) 1K1C rats (H) and the corresponding age-matched, sham-operated controls (S). Values are means ± SEM from seven different animals in each group. Key: (*) statistically significant ($P < 0.05$) versus sham-operated.

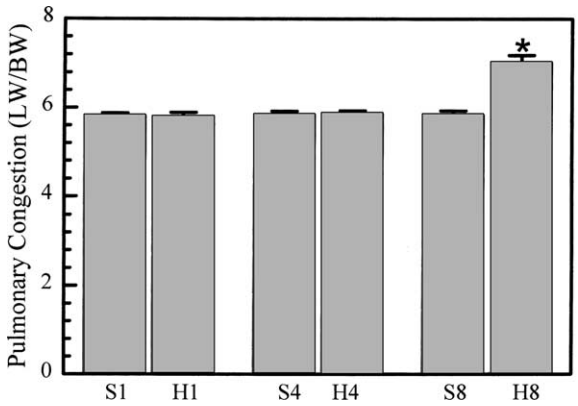

Fig. 3. Ratio of lung weight (LW) to body weight (BW) in 1-week (S1, H1), 4-week (S4, H4), and 8-week (S8, H8) 1K1C rats (H) and the corresponding age-matched, sham-operated controls (S). Values are means ± SEM from seven different animals in each group. Key: (*) statistically significant ($P < 0.05$) versus sham-operated.

Ca^{2+}-uptake was found to be unchanged in 1K1C rats at 1 week (15.23 ± 1.3 vs 14.03 ± 1.0, N = 7), significantly increased at 4 weeks (22.63 ± 1.8 vs 14.63 ± 1.1, N = 7), and significantly decreased at 8 weeks (8.63 ± 0.7 vs 14.93 ± 1.3, N = 7) post-surgery compared to the age-matched, sham-operated controls (Fig. 4). Furthermore, the affinity (μM) of the SR Ca^{2+}-uptake in 1K1C rats was unchanged at 1 week (0.53 ± 0.03 vs 0.50 ± 0.03, N = 7), significantly increased at 4 weeks (0.54 ± 0.03 vs 0.42 ± 0.02, N = 7), and significantly decreased at 8 weeks (0.52 ± 0.04 vs 0.69 ± 0.05, N = 7) post-surgery compared to the age-matched controls (Fig. 4).

3.3. Quantitation of CAA expression level

The expression level of immunodetectable CSQ did not change in the LV homogenate obtained from 1K1C rats or sham-operated controls at 1, 4, and 8 weeks post-surgery (Fig. 5). Further, this protein level remained unchanged between 1K1C rats and the sham-operated controls during the development of LV hypertrophy at 1, 4, or 8 weeks post-surgery. Since the amount of CSQ was not altered during the development of LV hypertrophy, the expression level of CAA was normalized to the total amount of protein loaded on the gel. At 1 week post-surgery, the immunodetectable

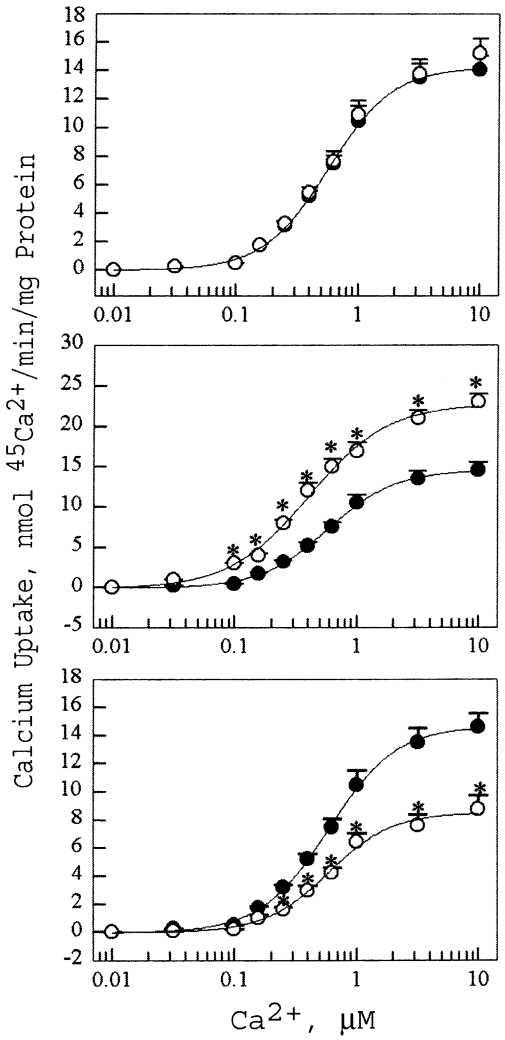

Fig. 4. Oxalate-dependent SR Ca^{2+}-uptake as a function of free Ca^{2+} in the LV myocardium of 1K1C rats and sham-operated rats at 1 week (top), 4 weeks (center), and 8 weeks (bottom). Ca^{2+}-uptake is expressed as nanomoles $^{45}C^{2+}$ per minute per milligram protein. Closed circles are for sham-operated rats and opened circles for 1K1C rats. Values are means ± SEM from seven different animals in each group. Key: (*) statistically significant ($P < 0.05$) compared to age-matched, sham-operated controls.

protein level of CAA in 1K1C rats did not change (1.30 ± 0.1 vs 1.40 ± 0.1 densitometric units/5 μg protein) compared with the sham-operated controls (Figs. 5 and 6, left panel). At 4 weeks post-surgery, CAA expression

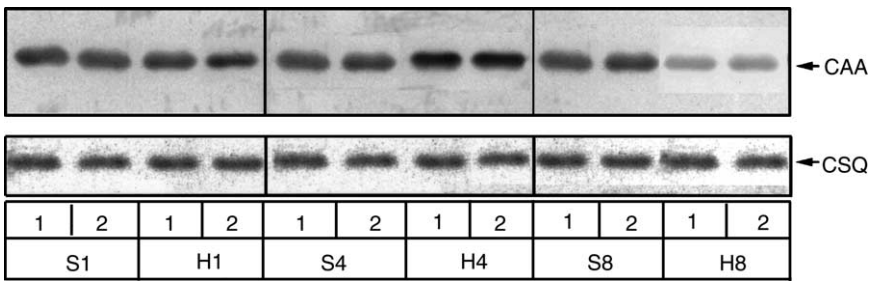

Fig. 5. Western blot exhibiting immunodetectable sarcoplasmic reticulum CAA and CSQ levels in LV myocardium of 1K1C and sham-operated rats. Using antibody, the level of CAA or CSQ was detected in the SDS-extract prepared from two LV specimens from 1-week (S1, H1), 4-week (S4, H4), and 8-week (S8, H8) 1K1C rats (H) and the corresponding age-matched, sham-operated controls (S).

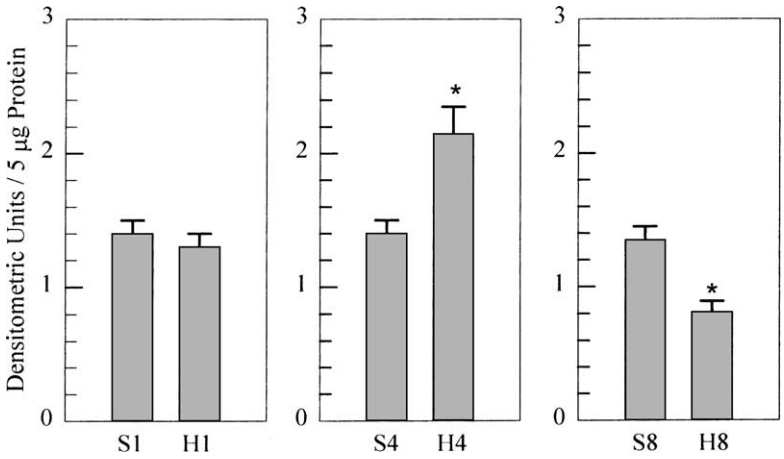

Fig. 6. Densitometric analysis of the Ca^{2+}-ATPase band from western blots of extracts of MV from 1K1C rats at 1 week (H1), 4 weeks (H4), and 8 weeks (H8) and of age-matched, sham-operated controls (S1, S4, and S8). Values are means ± SEM from seven different animals in each group. Key: (*) statistically significant ($P < 0.05$) compared with control.

significantly increased from 1.40 ± 0.1 densitometric units/5 µg protein observed in sham-operated control rats to 2.15 ± 0.2 densitometric units/5 µg protein in 1K1C rats (Figs. 5 and 6, center panel). By 8 weeks post-surgery, the CAA expression level significantly declined (0.84 ± 0.05 vs 1.37 ± 0.1 densitometric units/5 µg in 1K1C rats vs controls) (Figs. 5 and 6, right panel). Changes in the expression level of CAA among the sham-operated control groups were not evident.

4. Discussion

In the present study, we report that 1K1C rats at 1 and 4 weeks post-surgery developed LV hypertrophy without pulmonary congestion, whereas at 8 weeks they developed both LV hypertrophy and pulmonary congestion. Further, SR Ca^{2+}-uptake and CAA expression did not change at 1 week, increased at 4 weeks, and decreased at 8 weeks post-surgery in 1K1C rats compared with their age-matched, sham-operated controls. The increase in Ca^{2+}-uptake and CAA expression suggests a compensatory response at the cellular level to LV hypertrophy at 4 weeks, while the decline at the later stage (8 weeks) indicates the transition to heart failure.

Depressed SR Ca^{2+}-uptake has been observed in explanted failed human hearts [9] and in hearts from animals with heart failure produced by various methods [9,10]. It is controversial whether this abnormality exists in adaptable, compensated hypertrophied hearts [10]. Several studies describe alterations in SR Ca^{2+} handling during the progression of cardiac hypertrophy induced by pressure-overload due to aortic-banding [11–18], myocardial ischemia produced by left coronary artery ligation [19–23], and volume-overload generated by mitral regurgitation [24,25]. In agreement with previous studies [26], the present work shows that LV hypertrophy develops within 1 week (mild) and continues to increase at 4 (moderate) and 8 (severe) weeks in 1K1C rats. SR function during the development of LV hypertrophy in 1K1C rats was not reported earlier. In the present study, we found that the maximal velocity of SR Ca^{2+}-uptake remained unaltered in mild, increased in moderate, and decreased in severe LV hypertrophy. Although significant increases in the LVW/BW ratio occurred, SR Ca^{2+}-uptake did not change during mild LV hypertrophy at any time. Our findings in this model of LV hypertrophy are in contradiction to the findings of those who reported depressed SR function upon myocardial infraction [19–23], but agree with those who reported increased SR function after aortic constriction [11,12]. Although we did not assess LV function of hypertrophied hearts, the results of our study on pulmonary congestion suggest that LV hypertrophied hearts at 1 and 4 weeks are in a compensated state, but hypertrophied hearts at 8 weeks are in a decompensated, congested, and LV failure state. These observations are further corroborated by observing no changes in SR Ca^{2+}-uptake in LV hypertrophied myocardium at 1 week, increased SR Ca^{2+}-uptake at 4 weeks, and decreased SR Ca^{2+}-uptake at 8 weeks post-surgery in 1K1C rats. Changes in the SR function during the development of LV hypertrophy produced by a shunt operation in rats [24] are also reported in which the mRNA level of CAA was determined. Although in that study SR Ca^{2+}-uptake was not determined and, therefore, a direct comparison cannot be made, rat cardiac function increased at 7 days, returned to baseline levels at 21 days, and significantly decreased at 35 days after the shunt operation. Further, the level of CAA mRNA significantly decreased at both 21 and 35 days after the shunt operation, whereas PLB mRNA levels decreased only at 35 days. These findings suggest that the decrease in the CAA mRNA level preceded the development of cardiac dysfunction. Our results also suggest that changes in SR

Ca^{2+}-uptake in 1K1C rats are both quantitative and qualitative at 4 and 8 weeks post-surgery. The qualitative changes may be due, in part, to alteration in the CAA/PLB ratio and/or to the phosphorylation level of PLB during the development of LV hypertrophy. In failing hearts, both the CAA/PLB ratio and the phosphorylation level of PLB have been reported to be reduced [20]. Thus, increases in the affinity of SR Ca^{2+}-uptake for Ca^{2+} in 1K1C rats at 4 weeks may be due to an increase in the CAA/PLB ratio or an increased phosphorylation state of PLB or both and reduced SR Ca^{2+}-uptake affinity for Ca^{2+} in 8-week 1K1C rats may be due to a decrease in the CAA/PLB ratio and phosphorylation level of PLB. Although in the present study, the level of PLB was not measured, the level of CAA was determined. In 1K1C rats, unchanged SR Ca^{2+}-uptake at 1 week post-surgery was associated with an unaltered CAA level, increased SR Ca^{2+}-uptake at 4 weeks was associated with an increased amount of CAA, and reduced SR Ca^{2+}-uptake at 8 weeks was associated with a reduced level of CAA. Our findings are in agreement with those who showed increased CAA expression in aortic-banded LV hypertrophied hearts [11,12].

In summary, our findings demonstrate that Ca^{2+}-uptake increases early in the development of LV hypertrophy, and then declines in the later stages. These data parallel the progression of heart failure, whereby the myocardium first adapts to an increase in workload by developing a compensatory response, then later exhibits abnormalities in calcium handling and decreased contractility. The present study proposes an explanation for these changes at the cellular level.

Acknowledgments

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