Original Paper

Changes in T-Tubules and Sarcoplasmic Reticulum in Ventricular Myocytes in Early Cardiac Hypertrophy in a Pressure Overload Rat Model

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Key Words

Cardiac hypertrophy • Calcium signaling • Pressure overload • Fluorescence recovery after photobleach • Sarcoplasmic reticulum • Transverse tubules

Abstract

Background/Aims: Pressure-overload (PO) causes cardiac hypertrophy (CH), and eventually leads to heart failure (HF). HF ventricular myocytes present transverse-tubules (TT) loss or disarrangement and decreased sarcoplasmic reticulum (SR) density, and both contribute to altered Ca^{2+} signaling and heart dysfunction. It has been shown that TT remodeling precedes HF, however, it is unknown whether SR structural and functional remodeling also starts early in CH. **Methods:** Using confocal microscopy, we assessed TT (with Di-8-ANNEPS) and SR (with SR-trapped Mag-Fluo-4) densities, as well as SR fluorophore diffusion (fluorescence recovery after photobleach; FRAP), cytosolic Ca^{2+} signaling and *ex vivo* cardiac performance in a PO rat hypertrophy model induced by abdominal aortic constriction (at 6 weeks). **Results:** Rats developed CH, while cardiac performance, basal and upon β -adrenergic stimulation, remained unaltered. TT density decreased by ~14%, without spatial disarrangement, while SR density decreased by ~7%. More important, FRAP was ~30% slower, but with similar maximum recovery, suggesting decreased SR interconnectivity. Systolic and diastolic Ca^{2+} signaling and SR Ca^{2+} content were unaltered. **Conclusion:** SR remodeling is an early CH event, similar to TT remodeling, appearing during compensated hypertrophy. Nevertheless, myocytes can withstand those moderate structural changes in SR and TT, preserving normal Ca^{2+} signaling and contractility.

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Introduction

Cardiac hypertrophy occurs in response to persistent physiological or pathological hemodynamic challenges, and heart and cell enlargement are accompanied by structural and functional remodeling at the whole heart, interstitial, subcellular and molecular level [1]. Pressure overload (PO) is one of the main stimuli that trigger the remodeling observed in pathological cardiac hypertrophy, which eventually progresses to heart failure (HF) [1-3]. It is well known that in hypertrophied cells from HF, the transverse tubules (TT), the highly specialized system of sarcolemmal invaginations in ventricular myocytes, undergoes remodeling, which includes disarrangement or disappearance of tubules [4-11]. Since TT contains most of the L-type Ca^{2+} channels, essential for the excitation-contraction coupling (ECC) [12], TT remodeling in HF affects ECC by delaying recruitment of clusters of Ca^{2+} release channels (RyR) of the sarcoplasmic reticulum (SR) by the sarcolemmal Ca^{2+} current, or failing to activate them [4, 5, 8, 13]. Overt TT remodeling affects the spatial and temporal homogeneity of the systolic Ca^{2+} transient, which appears with blunted amplitude and slower time course, and all these impair the magnitude and rate of force development [4, 5, 8, 11]. Nevertheless, TT remodeling is a progressive process and it has been suggested that it precedes HF [11, 14]. Wei et al. showed that TT remodeling, in a PO model induced by thoracic aortic constriction (TAC), was apparent during compensated cardiac hypertrophy [14], while Shah et al. showed that increased TT alterations in uncompensated cardiac hypertrophy, assessed in the chronic spontaneously hypertensive rats (SHRs), correlated with worsening of cardiac function [11]. However, it is not known how early TT remodeling starts during PO-induced hypertrophy, and whether it is accompanied by remodeling of the closely associated SR.

The SR network is continuous and uniformly distributed throughout the cell volume [15, 16], and diffusion of luminal Ca^{2+} allows homogenous diastolic SR Ca^{2+} concentration at the whole cell level [17], as well as rapid local replenishment following spontaneous release, [17-19]. These ensure uniform Ca^{2+} release during ECC and avoids regional variations of RyR activity, due to varying luminal Ca^{2+} -dependent regulation [20], which could enhance spontaneous Ca^{2+} release [21, 22].

A number of studies have shown plasticity of the SR under normal and abnormal conditions. A recent report showed that the SR is a highly dynamic organelle, particularly during development, with continuous network branching and fusion, as well as intra network domain migration, and these dynamic processes also occurred in adult myocytes, albeit at lower frequency [23]. Recent reports; one in a sheep model of tachypacing-induced HF [16] and others in rat [9] and human HF [10], showed a ~30% decrease in SR density, accompanied by other ultra structural changes. Furthermore, SR plasticity has also been documented in transgenic animal models; calsequestrin (CSQ) knock out (CSQ-KO) increased SR volume [24], while mutated CSQ (R33Q knock-in) caused a ~30% decrease in junctional SR (jSR) [25]. Conditional SERCA-KO caused a ~73% decrease in SR volume, while longitudinal TT largely increased, enhancing sarcolemmal Ca^{2+} entry [26]. Nevertheless, it has not been determined whether changes in SR density and SR interconnectivity occur during early stages of cardiac hypertrophy. Given the prominent role of the SR in intracellular Ca^{2+} cycling; removal, storage and release, structural changes in this organelle could potentially impact Ca^{2+} signaling and contractile performance. Here we assessed whether SR remodeling occurs in ventricular myocytes from a rat model of compensated cardiac hypertrophy induced by PO (at 6 weeks), and its possible repercussion in Ca^{2+} cycling. The relative SR density and network interconnectivity were assessed with confocal imaging of an SR-trapped fluorophore (Mag-Fluo-4) in permeabilized ventricular myocytes. SR interconnectivity was assessed by the extent and time course of luminal Mag-Fluo-4 diffusion (fluorescence recovering after photobleach; FRAP). We found in hypertrophied myocytes (cellular volume increased by ~78%), a modest, but significant, ~7% decrease in SR density. More important, there was a ~30% decrease in the rate of intra SR fluorophore diffusion, suggesting a decrease in SR network interconnectivity or increased network "tortuosity". These changes coincided

with a ~14% decrease in TT density (without altering its normal distribution pattern). Nevertheless, at this early stage of cardiac hypertrophy, both structural changes in SR and TT were not accompanied by alterations in intracellular Ca^{2+} signaling, since Ca^{2+} transients and spontaneous sparks, as well as the SR Ca^{2+} content, were unchanged. Furthermore, *ex vivo* cardiac performance, at basal level and upon β -adrenergic stimulation, was unaltered. The data suggest that in rat hearts with compensated hypertrophy, moderate alterations in SR density and in its interconnectivity, accompanied by a modest decrease in TT, may appear as early as six weeks after AAC.

Materials and Methods

Reagents

Chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA, unless indicated otherwise. All fluorophores were obtained from Life Technologies, Carlsbad, CA, USA.

Rat model of pressure overload

These studies were approved by the Internal Committee for Care and Handling of Laboratory Animals of the School of Medicine of the Tecnológico de Monterrey (Protocol number 2012-012), and were performed following the NIH guidelines. We induced PO cardiac hypertrophy by abdominal aortic constriction (AAC) in male Wistar rats (250-350 g). Rats were anesthetized with pentobarbital sodium (30 mg/kg body weight, I.P.) and the surgical procedure was performed by laparotomy and exposure of the abdominal aorta proximal to the renal arteries, as described by others [27, 28]. In the AAC group, the constriction was performed between the left renal artery and the superior mesenteric artery with prolene thread and a G-22 needle as a guide, which reduced the lumen of the aorta by 65% [29], while age-paired sham-operated rats served as controls (Sham). Animals were fed with standard rat chow and provided water *ad libitum*. After 6 weeks of the surgical procedure, the animals were sacrificed and the *ex vivo* and *in vitro* studies were performed.

Isolated heart mechanical performance

Animals were anaesthetized with pentobarbital sodium (80 mg/kg body weight, I.P.), hearts were excised and mounted on a Langendorff apparatus, perfused retrogradely (12 ml/min) with Krebs-Henseleit (K-H) buffer at 37°C and bubbled with 95% O_2 / 5% CO_2 gas. K-H buffer had the following composition (in mM): 125 NaCl, 5.4 KCl, 1 MgCl_2 , 0.5 NaH_2PO_4 , 25 NaHCO_3 , 2.5 CaCl_2 , 11 glucose and 0.0001 octanoate [30]. A latex balloon connected to a pressure transducer was inserted into the left ventricle and filled with saline solution. Data were recorded with a Data-Trax acquisition system (World Precision Instruments, Sarasota, FL, USA). The hearts were perfused with K-H buffer and monitored for 30 min. The myocardium performance index (MPI) was obtained from the analysis of 5 min of contraction data. MPI represents the product of Left Ventricular Developed Pressure $\times$ Heart rate ($\text{LVDP} \times \text{HR}$; mm Hg $\times$ heart beats $\times \text{min}^{-1}$). Cardiac performance in response to stress was assessed by perfusing the hearts with K-H solution containing 100 nM of the synthetic β -adrenergic agonist isoproterenol (ISO) during 10 min.

Acutely Isolated Ventricular Myocytes

Myocytes isolation was performed following previously described procedures [31]. Briefly, hearts were excised from rats anaesthetized (as described above), and the heart was Langendorff-perfused with collagenase type II (1 mg/ml; Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C. Afterwards, the ventricles were removed and cells were mechanically dissociated. Myocytes were stored in a normal Tyrode (NT) solution containing (in mM): 130 NaCl, 5.4 KCl, 0.5 MgCl_2 , 25 HEPES, 22 glucose, 0.33 NaH_2PO_4 and 1 CaCl_2 (pH 7.4), at room temperature. Experiments were performed in quiescent rod-shaped cells within 6 hours of isolation.

Cell volume and T-Tubules imaging

Acutely isolated rat myocytes were incubated at room temperature in NT containing calcein acetoxymethyl ester (AM; 5 μM) to assess cell volume or with Di-8-ANEPPS (10 μM) to assess T-Tubules. After 20

min of fluorophore incubation, the cells were washed with fluorophore-free NT and confocal imaging was performed after 30 min with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), equipped with a HCX PL APO 40 $\times$, 1.3 NA, oil immersion objective. Calcein or Di-8-ANEPPS were excited at 488 nm with an Argon laser. Emission was collected at 500-600 and >600 nm, for calcein and Di-8-ANEPPS, respectively. Confocal images of 512 $\times$ 512 pixels were acquired in the XY scan mode at 400 Hz, with a pinhole optimized for a resolution of 0.4 μ m in the focal plane and <1 μ m in the z-axis. Images were digitized at 8 bit resolution, with a pixel size of 210-360 nm for TT imaging and 230-430 for cell volume (depending on cell size).

For cell volume assessment, stacks of confocal sections separated by 1 μ m in the z-axis covering the whole cell depth were acquired. Image processing and data analysis for cell volume was performed as described [32] using the public domain software "ImageJ" (<http://rsb.info.nih.gov/ij/>). Briefly, the cell area for each confocal plane was estimated by the pixel size multiplied by the number of pixels above a threshold, determined from the histogram of fluorescence intensity distribution. Cell volume was estimated by multiplying the area from each confocal section by the z step distance between confocal slices.

For the T-Tubule recording, we selected the center of the cell (in the z-axis), to avoid stained cell debris at the bottom of the chamber and the surface membrane, and acquired a stack of 10 confocal sections spaced every 100 nm along the z-axis. Each confocal section was the average of 4 frames from the same position, which allowed better structural resolution.

For the T-Tubule density analysis we used the full stack of 10 images, and it was performed as previously described [33, 34]. Briefly, a region of interest (ROI) was outlined inside the whole cell. The ROI was drawn by hand and excluded the surface sarcolemma and nuclei. To estimate the area containing Di-8-ANEPPS fluorescence, corresponding to the T-tubules, a threshold level was determined and data were analyzed as described above for cell volume. Results are presented as the relative "T-Tubule index" (TT index), which was obtained by dividing the total Di-8-ANEPPS fluorescence containing area by the total cell area from the 10 confocal slices, and normalized to Sham (%). To assess the longitudinal spacing between TT (T-Tubule distribution) and overall organization, a Fast Fourier transform (FFT) was used to convert the two dimensional images from the spatial domain in the longitudinal axis into the frequency domain [4, 11, 26]. For that purpose, the fluorescence profiles of two defined ROIs of 60 $\times$ 0.5 μ m positioned in different locations along the longitudinal axis of the cell were processed to provide an estimate for each cell. Data are displayed as the power vs. distance.

Sarcoplasmic reticulum density assessed in saponin-permeabilized cells loaded with Mag-Fluo-4

Myocytes were incubated with the low affinity Ca²⁺ fluorescent probe Mag-Fluo-4 AM at 30° C during 30 min, as described previously to favor fluorophore accumulation within the ER/SR lumen [35-38]. The fluorophore-loading solution contained (in mM) 0.1 CaCl₂, 0.0075 Mag-Fluo-4 (AM), 130 NaCl, 5.4 KCl, 25 HEPES, 1 MgCl₂, 0.3 NaH₂PO₄ and 20 glucose (pH 7.4). Afterwards, cells were washed with a fluorophore-free solution, kept at room temperature and used within 1 h.

To remove remaining cytosolic Mag-Fluo-4, myocytes were permeabilized with saponin as previously described [39]. Briefly, myocytes were incubated with a "relaxing" solution, containing (in mM) 100 K-aspartate, 20 KCl, 3 MgATP, 0.1 EGTA, 0.0001 free Ca²⁺, 1 free Mg²⁺, 20 HEPES, 3 K-Glutamate and 3 Malic acid (pH 7.4). After 1 min, the relaxing solution was replaced with a saponin-permeabilizing solution, containing (in mM, unless otherwise indicated) 100 K-aspartate, 20 KCl, 3 MgATP, 0.1 EGTA, 0.0001 free Ca²⁺, 1 free Mg²⁺, 20 HEPES, 3 K-glutamate, 3 Malic acid, 5 phosphocreatine di-Na, 5 U/ml creatine-phosphokinase, 2% polyvinylpyrrolidone (PVP) and 0.007% saponin (pH 7.2). After 30-60 s, this solution was replaced by a saponin-free "internal" solution, containing (in mM, unless indicated): 100 K-aspartate, 20 KCl, 3 MgATP, 0.5 EGTA, 0.00002 free Ca²⁺, 1 free Mg²⁺, 20 HEPES, 3 K-glutamate, 3 Malic acid, 5 phosphocreatine di-Na, 5 U/ml Creatine-phosphokinase and 2% PVP (pH 7.2). Experiments were performed within 15 min after permeabilization. Ca²⁺ and Mg²⁺ concentrations were calculated with the software Maxchelator [40].

Confocal images of 512 $\times$ 512 pixels were acquired in the XY scan mode, using the 488 nm Argon laser as the excitation wave length. Emission and other settings were as described above for cell volume. Stacks of three confocal sections separated by 100 nm in the center of the z-axis were acquired. For SR density assessment a region of interest (ROI) was outlined along the cell boundary. SR density analysis and data presentation were performed as described for T-tubules.

Fluorescence recovery after photobleach (FRAP) of sarcoplasmic reticulum-trapped Mag-Fluo-4

Myocytes were loaded with Mag-Fluo-4 and permeabilized with saponin as described above. X-Y images were acquired every 10 s, and each image was acquired at 400 Hz. After basal fluorescence was established (first 2 images), an ROI in the center of the cell (in the X-Y plane; ROI-a) of approximately $10 \times 10 \mu\text{m}$ was photobleached using the 488 nm Argon laser line with 100% transmission (basal fluorescence and recovery images were acquired using 8% transmission for the excitation laser to minimize unintended Mag-Fluo-4 bleaching). The selected ROI-a was scanned 2 times, followed immediately by acquisition of a sequence of full X-Y images (each image was acquired every 10 s) to establish the extent of photobleach and time course of recovery. Data acquisition lasted 300 s. ImageJ was used to obtain the fluorescence profile of photobleached ROI-a and the parallel fluorescence decrease of a second ROI (ROI-b) situated $50 \mu\text{m}$ away, in the longitudinal axis, from the bleached region. Mag-Fluo-4 FRAP time course data were represented as $\Delta F/F_0$ (%), where F is fluorescence at any given time and F_0 is basal fluorescence before bleaching.

Confocal imaging of cytosolic Ca^{2+}

Myocytes were incubated in NT solution containing Fluo-4 AM ($10 \mu\text{M}$) during 30 min, and washed afterwards with a fluorophore-free NT solution. Linescan images were recorded along the longitudinal axis of the cell, avoiding the nuclei, at 400 Hz. Excitation, emission and other imaging parameters, were as described for cell volume and SR density recording. Ca^{2+} transients were evoked with a field-stimulator (MYP100 MyoPacer, IonOptix LLC, Milton, MA, USA). Diastolic Ca^{2+} sparks were acquired during 2-3 s after evoked Ca^{2+} transients returned to basal level.

The SR Ca^{2+} content was assessed by the amplitude of the cytosolic Ca^{2+} transient in response to rapid caffeine (10 mM) application after a conditioning field stimulation train [41]. Fluo-4 fluorescence was presented as normalized F/F_0 , as described above. Ca^{2+} sparks were characterized according to established criteria [42] using the ImageJ plugin Sparkmaster [43].

Statistics

Data were presented as mean $\pm$ standard error of the mean (SEM). n represents the number of animals or cells studied. Unpaired Student's t-tests were performed and a $P < 0.05$ was considered significant. Data processing and statistical analysis were performed with Microsoft Excel (2007, Redmond, WA, USA) and GraphPad Prism (V. 2.0, La Jolla, CA, USA). Graphs were prepared using Systat Sigma Plot (V. 12, San Jose, CA, USA).

Results

Whole heart and ventricular myocytes hypertrophy in AAC rats

At 6 weeks following AAC surgery, we found a significant $\sim 23\%$ increase in the heart/femur weight ratio (from 0.92 ± 0.05 to $1.13 \pm 0.02 \text{ g} \times \text{g}^{-1}$, for Sham and AAC hearts, respectively, $P < 0.05$) (Fig. 1A). Cardiac hypertrophy was confirmed at the cellular level by assessing ventricular myocytes volume using confocal imaging. Figure 1B shows representative images of Sham and AAC ventricular myocytes loaded with calcein. Pooled data showed (Fig. 1C) a $\sim 78\%$ volume increase in AAC ventricular myocytes (from $31.5 \pm 1.3 \text{ pl}$ to $56.0 \pm 4.4 \text{ pl}$, for Sham and AAC, respectively, $P < 0.05$).

Ex vivo cardiac contractility performance in AAC rats

Cardiac contractility performance in AAC and Sham rats was assessed in isolated hearts under basal conditions. Pooled data of MPI assessed during 30 min for both heart types is shown in Fig. 2A. We found no difference in MPI in AAC hearts, compared to Sham ($P > 0.05$). Furthermore, we assessed AAC and Sham MPI response to β -adrenergic stimulation. Fig. 2B shows. Pooled data of the % increase in MPI at 10 min showed no significant difference in both heart types ($P > 0.05$). These data suggest that at 6 weeks the AAC hearts were in compensated hypertrophy.

Fig. 1. Cardiac hypertrophy assessed at the organ and cellular level in AAC rats. (A) Pooled data of the heart to femur weight ratio in Sham and AAC rats after 6 weeks of surgery (n=5 animals for each group). (B) Confocal sections from representative ventricular myocytes loaded with calcein. (C) Mean cell volume (pl) obtained from stacks of confocal sections acquired in the z axis (n=27 cells/4 rats for Sham; 21 cells/4 rats for AAC). White bar in the AAC image represents 10 μ m and both cells share the same scale.

Fig. 2. *Ex vivo* mechanical performance in AAC hearts. (A) Basal mechanical performance index in AAC and Sham hearts (n=3 for Sham and AAC). MPI units are mm Hg $\times$ heart beats $\times$ min⁻¹. (B) Normalized data of MPI of Sham and AAC hearts upon ISO exposure (n=5 for Sham and AAC).

Structural changes in T tubules in AAC ventricular myocytes

It is well established that in HF the structural changes in TT contribute to altered Ca^{2+} signaling [4, 5, 11] and heart performance [11, 14]. We aimed to determine whether the AAC cells, in early stages of cardiac hypertrophy (at 6 weeks), presented TT alterations, as reported by Wei et al., after ~ 9 weeks in the more aggressive TAC rat model [14]. Figure 3A shows representative confocal images of both, Sham and AAC ventricular myocytes incubated with the lipophilic fluorophore Di-8-ANEPPS. In both cell types the TT system appears as highly organized transversal bands spaced periodically along the longitudinal axis of the cell. From z-stacks of 10 images per cell, as those shown in Fig. 3A, we assessed TT density (TT index) and TT longitudinal spacing, and we found a modest, but significant, decrease in AAC ventricular myocytes, compared with Sham ($\sim 14\%$; $P < 0.05$, Fig. 3B). The longitudinal TT distribution, assessed with a FFT, did not show differences in both cell types (Fig. 3C). The main peak of the power distribution was used to compute TT longitudinal periodicity, and corresponded to $1.81 \pm 0.01 \mu\text{m}$ for Sham and $1.82 \pm 0.03 \mu\text{m}$ for AAC ($P > 0.05$), which are in agreement with the normal TT distribution found in previous studies [4, 6, 14, 44]. Furthermore, there were no changes in the power of the main peak, which is an index of the regularity of longitudinal TT distribution (2.24 ± 0.01 and 2.27 ± 0.09 , for Sham and AAC, respectively, $P > 0.05$).

Structural changes in the sarcoplasmic reticulum in hypertrophied ventricular myocytes

Changes in SR structure and function in failing myocytes has been an intriguing issue, with important potential consequences for Ca^{2+} signaling, mechanical and electrical myocyte

Fig. 3. T-tubule density and longitudinal distribution in AAC ventricular myocytes. (A) Representative confocal images from di-8-ANEPPS-stained Sham and AAC ventricular myocytes. Sections in the right represent a magnification of the regions within the rectangles in the main images. (B) Pooled data for the TT index in both cell types (n=23 cells/4 animals for Sham; 26 cells/4 animals for AAC). White bar in the AAC image represents 10 μ m, and both cells share the same scale. (C) FFT power distribution (arbitrary units) along the longitudinal axis from di-8-ANEPPS-stained Sham and AAC cells.

Fig. 4. Sarcoplasmic reticulum density in AAC ventricular myocytes. (A) Representative confocal images of intra-SR Mag-Fluo-4 fluorescence in permeabilized Sham and AAC ventricular myocytes. Sections in the right represent a magnification of the regions within the rectangles in the main images. (B) Pooled data for the relative SR density (SR-D (%)) in both cell types. (n=19 cells/3 animals for Sham; 18 cells/4 animals for AAC). White bar in the AAC image represents 10 μ m, and both cells share the same scale.

performance [17], as well as for protein processing and SR-mediated myocyte apoptosis [45, 46]. Recent structural studies in myocytes from HF in human and animal models [9, 10, 16] have shown decreased SR density, accompanied by other ultra structural dyad changes. However, it is unclear whether those structural changes appear late during HF progression or are events that could appear during early stages of cardiac hypertrophy. Furthermore,

Fig. 5. Fluorescence recovery after photobleach of intra-SR Mag-Fluo-4 in AAC permeabilized ventricular myocytes. (A) Confocal images of FRAP of the intra-SR Mag-Fluo-4 at 0, 50, 100 and 150 s after photobleach in a Sham and an AAC cell. Letters a and b within the upper images indicate the ROIs selected for assessing fluorescence recovery and decay, respectively. (B) Time course of FRAP for the Sham and AAC ventricular myocytes. (C) Time course of fluorescence decay of Mag-Fluo-4 at 50 μm away from the bleached area.

those previous studies did not provide data about the status of the network interconnectivity resulting from the structural SR remodeling. Therefore, we assessed the relative SR density and the integrity of the SR network interconnectivity during compensated cardiac hypertrophy in AAC ventricular myocytes. For this purpose, we loaded the SR lumen with the low affinity Ca^{2+} indicator Mag-Fluo-4, followed by Saponin permeabilization of the sarcolemma. Figure 4A shows representative confocal images of the SR for Sham and AAC cells. Pooled data (Fig. 4B) shows a small, but significant decrease ($\sim 7\%$) in relative SR density in AAC ventricular myocytes ($P < 0.05$). SR network integrity was assessed with FRAP of Mag-Fluo-4 (Fig. 5). From those recordings, we evaluated both; Mag-Fluo-4 fluorescence recovery (in ROI-a), as well as fluorescence decrease in a distant region (in ROI-b; 50 μm away in the longitudinal axis; Fig. 5A). Figure 5B and C shows the time course of fluorescence change for ROI-a and ROI-b, respectively, for the Sham and the AAC ventricular myocytes shown in Fig. 5A. The time course of recovery in each cell type is apparently faster than that of decay. This is because the fluorescence recovery in the ROI-a depends on fluorophore diffusion from all adjacent regions; longitudinal as well as transversal. Single exponential fitting allowed determining the extent and time constant of fluorescence recovery (τ_{recovery}) in both cell types. Pooled data for this analysis is shown in Fig. 6. We found a significant increase ($\sim 44\%$) in τ_{recovery} in AAC cells (30.0 ± 4.4 and 43.2 ± 4.8 s, for Sham and AAC, respectively, $P < 0.05$; Fig. 6B). However, the maximum fluorescence recovery, assessed after 300 s, was similar in both cell types (42.3 ± 1.4 and $40.7 \pm 1.2\%$, for Sham and AAC, respectively, $P > 0.05$; Fig. 6C). Pooled data for the time constant of decay (τ_{decay}) in the ROI-b (Fig. 6E) showed a $\sim 82\%$ increase in AAC cells (145.9 ± 21.5 and 265.6 ± 46.6 s, for Sham and AAC, respectively, $P < 0.05$). Nevertheless, the maximum decay of Mag-Fluo-4 fluorescence after 300 s was similar in both cell types (67.6 ± 2.6 and $71.8 \pm 2.8\%$, for Sham and AAC, respectively, $P > 0.05$; Fig. 6F).

Fig. 6. Intra-SR Mag-Fluo-4 diffusion is slower in AAC ventricular myocytes. (A) Pooled data of the time course of intra-SR Mag-Fluo-4 fluorescence recovery in ROI-a. (B) Pooled data for the τ_{recovery} (n=14 cells/4 animals for Sham and AAC). (C) Maximum extent of fluorescence recovery at 300 s after bleach in the ROI-a. (D) Pooled data of the time course of Mag-Fluo-4 fluorescence decay in the ROI-b. (E) Pooled data for the τ_{decay} (n=12 cells/4 animals for Sham; 11 cells/4 animals for AAC). (F) Maximum fluorescence decay in the ROI-b at 300 s after bleaching.

Fig. 7. Field-stimulated Ca^{2+} transients in AAC ventricular myocytes. (A) Representative confocal images for the time course of the cytosolic Ca^{2+} transients in Fluo-4-loaded sham and AAC ventricular myocytes. Traces below each image illustrate the time course of the normalized spatial average fluorescence. Vertical scale bar in the Sham image represents 20 μm , and both cells share the same scale. (B) Derivative of the time course of $\Delta F/F_0$ (dF/dt ; %/ms) from the representative Ca^{2+} transients for Sham (black) and AAC (red). (C) Pooled data for dF/dt from both cell types. (D) Pooled data for the normalized Ca^{2+} transient peak amplitude ($\Delta F/F_0$) for Sham and AAC ventricular myocytes. (E) Time to 50% decay of the Ca^{2+} transient. (n=50 cells/ 4 animals for Sham; 47 cells/ 4 animals for AAC).

Cytosolic Ca^{2+} handling and SR Ca^{2+} content in AAC ventricular myocytes

We assessed systolic and diastolic Ca^{2+} handling by recording field-stimulated Ca^{2+} transients and spontaneous Ca^{2+} sparks in Sham and AAC ventricular myocytes loaded

Fig. 8. Spontaneous Ca^{2+} sparks in AAC ventricular myocytes. (A) Representative Ca^{2+} Sparks images from Sham and AAC ventricular myocytes loaded with Fluo-4. (B) Pooled data for Ca^{2+} sparks frequency. (C) Spark Amplitude ($\Delta F/F_0$). (D) Ca^{2+} spark duration, assessed as the time above the 50% level of the peak (full-duration-half-maximum; FDHM). Sham= 82 cells/ 4 animals, AAC= 69 cells/4 animals.

with cytosolic Fluo-4. Despite the decrease in TT and SR density, as well as the apparent diminished SR interconnectivity, all of which potentially could lead to cytosolic Ca^{2+} handling alterations, we found relatively normal systolic Ca^{2+} dynamics. Figure 7A shows representative line scan confocal images of Ca^{2+} transients and spatial average of normalized fluorescence (F/F_0) in Sham and AAC ventricular myocytes, and Fig. 7B shows the derivative of the time course of $\Delta F/F_0$ (dF/dt) in those cells, as an index of the SR Ca^{2+} release flux, and therefore an indication of SR Ca^{2+} release synchronization. Pooled data showed that dF/dt in AAC cells was not significantly different from Sham (11.06 ± 1.58 and 10.36 ± 0.95 %/ms, for Sham and AAC, respectively, $P > 0.05$; Fig. 7C). The Ca^{2+} transient peak amplitude was also unchanged (9.5 ± 0.2 and 9.8 ± 0.2 $\Delta F/F_0$, for Sham and AAC, respectively, $P > 0.05$; Fig. 7D). Finally, the time to 50% decay ($t_{50\%}$) of the Ca^{2+} transient, which is an index of cytosolic Ca^{2+} removal, mainly by SERCA, was unchanged in AAC (333 ± 11 and 313 ± 11 ms, for Sham and AAC, respectively, $P > 0.5$; Fig. 7E).

Figure 8A shows representative confocal images of Ca^{2+} sparks in AAC and Sham ventricular myocytes. Pooled data for Ca^{2+} sparks frequency (2.3 ± 0.2 and 2.2 ± 0.3 sparks/100 $\mu\text{m/s}$, in Sham and AAC, respectively, $P > 0.05$; Fig. 8B), spark amplitude (1.17 ± 0.03 and 1.13 ± 0.06 $\Delta F/F_0$, in Sham and AAC, respectively, $P > 0.05$; Fig. 8C) and spark duration (31.3 ± 1.0 and 30.4 ± 1.4 ms, in Sham and AAC, respectively, $P > 0.05$; Fig. 8D) were unchanged. Finally, as expected, according to the lack of alterations in systolic and diastolic Ca^{2+} handling, the SR Ca^{2+} content, assessed by rapid caffeine application, was similar in both cell types (8.1 ± 0.6 and 7.6 ± 0.5 $\Delta F/F_0$, in Sham and AAC, respectively. Sham = 15 cells/ 3 rats, AAC= 13 cells/ 3 rats. $P > 0.05$).

Discussion

Much attention has been paid on determining the structural and functional remodeling of ventricular myocytes during HF, particularly in the TT and SR [4, 5, 7, 9, 11, 16, 22, 45]. Nevertheless, HF is the final stage of many diseases, and therefore, remodeling at the whole heart, cellular and molecular levels is highly conspicuous, and clearly correlates with diminished cellular and whole heart performance. In contrast, data about the structure and function of TT and SR in early stages of cardiac hypertrophy are scarce. A couple of studies showed a decrease in TT and overall disarrangement in compensated [14] and

uncompensated [11] cardiac hypertrophy, suggesting that TT remodeling precedes whole heart dysfunction. However, it is not documented how early TT remodeling appears and whether SR remodeling also occurs in early hypertrophy stages. Here, we provided an assessment of both TT and SR remodeling, complemented with Ca^{2+} signaling studies, in ventricular myocytes from rats with compensated cardiac hypertrophy after six weeks of AAC. AAC is a well established PO-induced model of cardiac hypertrophy, where the progression of the syndrome can be clearly followed; from compensated to uncompensated stages and overt HF [47]. This is in contrast with other rodent PO-models (e.g. TAC [14]), where uncompensated hypertrophy and HF appear within 8-12 weeks ([14]) or models of cardiac infarct (e.g. coronary artery ligation, [48]), where HF develops within 10 weeks [48], thereby complicating the study of the gradual changes likely to occur in chronic diseases (e.g. hypertension and aortic stenosis). Capasso et al. [47] reported that, in the AAC model, it is necessary to prolong the PO stimuli until 8 months to observe uncompensated cardiac hypertrophy.

At 6 weeks after AAC we confirmed heart hypertrophy (Fig. 1A), similar to what others have reported starting as early as four weeks [27-29, 49, 50], while cardiac performance, assessed *ex vivo*, in basal and upon β -adrenergic stimulation, remained unaltered (Fig. 2). Cellular hypertrophy was conspicuous (Fig. 1C), while TT density modestly decreased without spatial disarrangement (Fig. 3). Moreover, a small decrease in SR density was also found (Fig. 4), and FRAP data showed slower SR fluorophore diffusion (Figs. 5 and 6), although the maximum fluorescence recovery was unchanged (Fig. 6C), suggesting decreased SR interconnectivity or increased path tortuosity. Nevertheless, systolic and diastolic Ca^{2+} signaling were unchanged (Fig. 7 and 8).

It has been described that in early stages of PO-induced cardiac hypertrophy, the ventricular myocyte volume enlargement is accompanied by a larger increase in contractile myofilaments, TT and SR membranes [51]. The larger increase in TT membrane compensates for the smaller increase in surface sarcolemma, keeping the total sarcolemma to volume ratio constant, which is essential for cell electrophysiology [51]. A larger cell also requires increased SR to match TT and contractile myofilaments, keeping the SR membrane to volume ratio constant, and maintaining ECC performance during this adaptive state [51, 52]. Nevertheless, if the insult persists, these adaptive changes become overwhelmed and the ventricular myocyte enters into the state of maladaptive hypertrophy [53] leading to HF; characterized by altered TT [4, 5, 7, 9, 11, 14, 22] and SR [9, 16], which contribute to cell dysfunction [1, 53]. In this study at 6 weeks following AAC we found a ~78% myocyte volume increase, while TT and SR densities modestly decreased. These suggest that the expected increases in TT and SR were unable to match cell volume enlargement [51], or that those structures had already started to obliterate. Changes in TT could occur at different levels; a decrease in tubule number, diameter or prolongation within the cell [12, 54]. TT remodeling also involves rearrangement from the normal organization at the Z line to the A band [4, 6, 44], without affecting the overall density [4]. Nevertheless, in our AAC myocytes FFT analysis yielded no change in TT distribution along the longitudinal axis, and the spacing coincided with the normal sarcomere length (Fig. 3C) [4, 6, 14, 44]. Optical limitations of confocal microscopy and data acquisition settings (limited number of slices in the z-axis) precluded analysis of TT diameter or depth projection into the cell. Nevertheless, systolic Ca^{2+} signaling (Fig. 7) and cardiac performance (Fig. 2) were unaffected, probably because the highly developed TT system in rat myocytes (constituting up to 21-33% of sarcolemma) [51] can withstand moderate decreases in TT, maintaining relatively normal ECC and heart performance. For TT alterations to have a clear effect in Ca^{2+} signaling and heart function, TT density has been reported to decrease by ~30-40% in human HF, pig and rat hypertrophy and HF models [5, 6], and ~24% in a tachypacing dog model [33]. Others have reported large TT disarrangements (assessed as ~40% decrease in overall TT integrity) [14] or were accompanied by major disruption in their distribution [9, 13] in TAC rodent HF models and in SHR with HF [4, 11]. Nevertheless, our data confirm that TT remodeling occurs in compensated cardiac hypertrophy, appearing as early as six weeks after AAC, preceding

alterations in Ca^{2+} signaling and contractile dysfunction. Di-8 ANNEPS fluorescence density is a standard technique to assess TT density [4-6, 11, 14, 33, 48], and the small difference that we found between both cell types could have not resulted from differential membrane affinity for the fluorophore or from differences in membrane potential, since both cell types were in resting conditions and were quiescent, as evidenced by the lack of motion artifacts, which is essential for acquiring full z-stacks of two-dimensional images. Furthermore, Stilli et al. (2001) [49], using patch clamp reported unaltered resting membrane potential in AAC cells after 4 weeks.

The SR occupies ~4% of the cell volume [16, 19, 51], and it is composed of two interconnected domains; jSR, containing most of the RyR, and the free SR (fSR), consisting of narrow tubules connecting to jSR terminals, and containing most of SERCA [19]. We found a discrete ~7% decrease in SR density, which apparently affected network interconnectivity. In principle, a decrease in SR density could be due to a decrease in the number or size of jSR cisternae [9, 25] or in the number or diameter of fSR tubules. A decrease in the number of jSR terminals would affect the magnitude (as suggested by Venetucci et al. (2003) for a global decrease in SR density [55]) and homogeneity of the Ca^{2+} transient front, since Ca^{2+} release would not occur at the normal locations. Nevertheless, immunolocalization studies have shown that even after TT misplacement in HF myocytes, “orphan”, but functional RyR receptors remain at the Z line [4, 13]. Delayed orphan RyR activation is probably triggered by Ca^{2+} diffusion from nearby active sites [4, 13]. A decrease in jSR individual size would diminish the number of junctional RyR and the amount of local Ca^{2+} , and both should affect Ca^{2+} sparks and Ca^{2+} wave propagation [9, 13, 25, 56]. Nevertheless, the dF/dt of the Ca^{2+} transient and the properties of the spontaneous Ca^{2+} sparks in the AAC cells studied here were unchanged (Figs. 7B-C and 8). These seem reasonable considering the small SR density decrease documented above, which is in contrast with the ~30% decrease in jSR reported in a catecholaminergic polymorphic ventricular tachycardia model [25] and in the more aggressive TAC model [9], where substantial effects on Ca^{2+} signaling occurred.

On the other hand, a decrease in the number or diameter of fSR tubules might decrease connectivity with individual jSR and within the whole network, impacting SR Ca^{2+} diffusion. Normally, the connections between fSR and jSR display some variability, and the extent of connectivity of a given jSR cisternae with a fSR terminal determines the rate of jSR Ca^{2+} replenishment upon spontaneous Ca^{2+} release [17-19, 57], and therefore should determine RyR refractoriness. Unfortunately, we were unable to study jSR Ca^{2+} recovery, since Mag-Fluo-4 K_d (~70-80 μM) [58] does not allow to detect the small fluorescence decrease typical of jSR depletion [17, 19], and Fluo 5N (K_d ~400 μM [59]) hardly enters into the rat SR [55]. Nevertheless, a decrease in network connectivity should perturb SR Ca^{2+} diffusion [16], and this might cause heterogeneous SR Ca^{2+} distribution, which could alter the Ca^{2+} -dependent functional status of RyRs in different jSR terminals [21, 22]. jSR with higher Ca^{2+} would be more prone to release, creating pro arrhythmic foci [17, 21, 22, 55]. Preliminary experiments in AAC cells did not show a difference in the frequency of spontaneous Ca^{2+} waves, in cells bathed with normal external Ca^{2+} , and the rate of Ca^{2+} wave propagation slightly increased (~23%; $P=0.05$) (Not shown). Earlier observations in cells from a canine model of compensated hypertrophy showed slow Ca^{2+} waves despite of high Ca^{2+} spark frequency [22]. In these cells the overall SR Ca^{2+} content was low, and the high spark frequency was explained arguing that Ca^{2+} heterogeneity within the SR caused isolated jSR Ca^{2+} -overload, and that slower wave propagation occurred because a given spark could not easily trigger adjacent jSR cisternae with lower Ca^{2+} . Nevertheless, in our AAC cells, whole cell SR Ca^{2+} and spark properties were unchanged; therefore fewer sites would have abnormally low Ca^{2+} and should not hamper wave propagation.

While cytosolic Ca^{2+} signaling was unaffected, we found that the intra SR fluorophore diffusion was markedly slower (Figs. 5 and 6) in AAC ventricular myocytes. The τ_{recovery} increased by ~44%; while τ_{decay} of fluorescence 50 μm away (ROI-b) from the bleached area increased by ~82%. Nevertheless, the maximum extent of fluorescence recovery (~40%) was unchanged, and occurred with a parallel decrease of ~30% of the fluorescence at the

ROI-b. Since fluorophore diffusion from distant regions within the network still occurred, this suggests that the SR is not fragmented, however, whole cell SR interconnectivity apparently decreased. Nevertheless, the data do not allow determining whether this is due to decreases in the number of fSR connecting units or in the fSR tubules diameter; both of which could delay intra-SR fluorophore diffusion. Furthermore, increased tortuosity of the fSR-jSR connections would also delay diffusion [17, 19, 21]. Nevertheless, intra SR diffusion of Ca^{2+} should be affected less drastically than in the case of the fluorophore. This is because Mag-Fluo-4 diffusion is slower than that of Ca^{2+} , due to its large molecular size [17, 21], and because SR Ca^{2+} can be restored by SERCA, while FRAP strictly depends on intra SR diffusion of free fluorophore. Nevertheless, since during HF SERCA expression is reduced [60], then the effect of diminished interconnectivity on Ca^{2+} distribution could become more important than in compensated hypertrophy.

FRAP studies required that SR Ca^{2+} remained in steady state throughout the experiment, which was achieved in permeabilized cells. Furthermore, Mag-Fluo-4 should be saturated at steady state SR Ca^{2+} (~1 mM) and, for that reason; unintended small SR Ca^{2+} changes may not affect fluorescence recordings. Therefore, FRAP should reflect Mag-Fluo-4 diffusion. In pilot experiments (not shown), we found that caffeine application (10 mM) decreased Mag-Fluo-4 fluorescence by ~65-70%, and the fluorescence recovery upon caffeine removal was thapsigargin-sensitive (not shown), confirming that a large proportion of the fluorophore was trapped within the SR.

We conclude that in the AAC PO model, which has been used for decades for the study of compensated [28, 29, 49, 50] and uncompensated [47] cardiac hypertrophy, both TT and SR remodeling start early in the compensated stage of cardiac hypertrophy, without major repercussions in normal Ca^{2+} cycling and cardiac performance. However, further structural and functional remodeling in those structures, changes in the expression of genes codifying for contractile and Ca^{2+} -cycling proteins [4, 5, 9-11, 14, 16, 22], would accelerate the cardiac deterioration typical of HF.

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Disclosure Statement

None.

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