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Progression of myocardial remodeling and mechanical dysfunction in the spontaneously hypertensive rat

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LeGrice IJ, Pope AJ, Sands GB, Whalley G, Doughty RN, Smaill BH. Progression of myocardial remodeling and mechanical dysfunction in the spontaneously hypertensive rat. *Am J Physiol Heart Circ Physiol* 303: H1353–H1365, 2012. First published September 21, 2012; doi:10.1152/ajpheart.00748.2011.—The progression of hypertensive heart disease (HHD) to heart failure (HF) is associated with myocardial remodeling. Corresponding changes in three-dimensional organization of cardiac extracellular matrix have not been quantified or related fully to the development of HF. Spontaneously hypertensive rats (SHRs) and Wistar-Kyoto controls were studied at 3, 12, 18, and 24 mo. Hemodynamic and morphological data, brain natriuretic peptide levels, and echocardiography demonstrate four distinct disease stages: systemic hypertension, diastolic dysfunction, early systolic failure, and decompensated HF. Passive left ventricular (LV) pressure-volume relationships were determined in vitro. Transmural specimens from the anterior LV free wall were imaged using extended-volume confocal microscopy, and three-dimensional myocardial architecture was quantified. In SHRs, LV compliance was reduced at 12 mo and increased progressively thereafter. However, it was less than in controls for filling pressures <10 mmHg and not significantly different at ≥10 mmHg. Myocyte cross section was enlarged, with increased variability from 12 mo, while collagen fraction increased progressively. Perimysial collagen fraction remained unchanged with age, although endomysial collagen increased from 12 mo. Perimysial collagen between adjacent muscle layers fused at 12 mo and continued to thicken subsequently, while muscle layers became more dispersed and disordered. We conclude that LV dilatation, which accompanies decompensated HF in this model of HHD, is not due to LV “softening.” While perimysial (and endomysial) collagen networks are substantially remodeled, they are not dissolved, as has been proposed. We argue that progressive disruption of the laminar organization of LV myocardium may contribute to impaired systolic function in HHD.

hypertension; cardiomyopathy; connective tissue; perimysium; endomysium; myocardium; laminar architecture

LONG-STANDING HYPERTENSION triggers a series of changes in left ventricular (LV) geometry and myocardial architecture that are initially compensatory but contribute to an eventual progression toward heart failure (HF) (3, 11, 15). In the classical description of this process, early concentric LV hypertrophy leads to impaired LV filling and then to the development of decompensated HF, where LV dilatation is accompanied by an ongoing reduction in ejection fraction. Recent clinical analyses suggest that the progression of hypertensive heart disease (HHD) to end-stage HF can be more complex than this (14).

For instance, the characteristics of HHD vary with the origin and extent of hypertension, age, and ethnicity (15), and symptomatic HF with normal ejection fraction is observed in a significant subset of hypertensive patients (23). Even within the classical paradigm of disease progression, the mechanisms that underlie transition to decompensated HF are poorly understood.

The progression of HHD has been studied extensively in the clinical setting and also with animal models of hypertension (19). The latter include genetic models, such as the well-established spontaneously hypertensive rat (SHR) (5), and models in which chronic hypertension is induced by constriction of the thoracic aorta or renal arteries (19). These animal models have the advantage of replicating key features of the development of HHD over a relatively short and predictable time course.

At the tissue level, HHD is associated with myocyte hypertrophy and progressive fibrosis (3, 15). Type I collagen synthesis and collagen cross-linking within the extracellular matrix (ECM) are increased initially in HHD (11), driven by activation of the transforming growth factor- β signaling pathway. At the same time, tissue levels of matrix metalloproteinases (MMPs) are reduced with respect to their tissue inhibitors (TIMPs), and collagen turnover is decreased as a result (3, 17). MMP-to-TIMP ratios increase with the transition to decompensated HF, but interstitial collagen proliferation continues (17). It has been argued that LV dilatation occurs here in the presence of fibrosis, because specific profibrotic processes initiated in the first stages of HHD are reversed (11, 15). Degradation of type I collagen, dissolution of ECM cross-linking, and loss of perimysial and endomysial collagen networks have been raised as potential mechanisms (3, 27, 28), but the few studies that address this issue have produced contradictory results (18, 27, 36). Apparent discrepancies could be due to differences in disease end points, the animal models employed, or the variability of HHD in the clinical setting. In addition, most analyses of collagen distribution in HHD have been descriptive, rather than quantitative, and based on selected two-dimensional (2D) histological images. The cardiac ECM has a complex three-dimensional (3D) arrangement, and recent detailed studies (35) have demonstrated that perimysial collagen organization in the normal heart cannot be characterized repeatably from 2D image sections, unless they are referred to appropriate local structural axes.

This study describes a time-course study in which the myocardial remodeling associated with the development of HHD has been characterized systematically, for the first time as far as we are aware. This has been done with the SHR, a

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well-validated animal model of HHD that replicates the classic progression of the disease. Key features of myocyte architecture and 3D collagen organization were quantified at four distinct end points: early systemic hypertension, diastolic dysfunction, early systolic failure, and decompensated HF. Hemodynamic and morphological data, measures of systolic and diastolic LV function, and systemic natriuretic peptide responses were recorded throughout this process.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee of the University of Auckland and conforms to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23).

Animal model. SHR were studied at four separate end points: 3, 12, 18, and 24 mo; age-matched Wistar-Kyoto (WKY) rats were used as controls. A total of 55 animals were raised in each group, but 1 SHR and 4 WKY rats died or were euthanized and were not studied. SHR that demonstrated signs of end-stage HF between 18 and 24 mo were studied and included with the latter end point. At specified age end points, heart rate and systolic blood pressure were determined using the tail-cuff method (model 179, IITC Life Science). For measurement of type I collagen, LV tissue from a separate group of 24-mo-old SHR ($n = 8$) and WKY rats ($n = 4$) was collected, processed, and immunohistochemically labeled.

Study protocol. Rats were lightly anesthetized with tiletamine-zolazepam (30 mg/kg ip), and LV geometry and function were characterized at 3, 12, 18, and 24 mo by M-mode echocardiography (model HDI 7000, Philips, Bothell, WA) in the equivalent short-axis parasternal view using a 12.5-MHz neonatal probe at minimal depth and maximum sweep speed. The average of 6–10 beats is presented. Rats were anesthetized (5% halothane) 2–8 days later, and the thoracic cavity was opened. Heparin (100 IU/kg) was immediately injected into the LV and allowed to circulate, and 500 μ l of blood were withdrawn. The heart was excised, cooled in chilled saline, blotted dry, and weighed. It was then suspended on a Langendorff apparatus and perfused with Krebs-Henseleit solution (0.1 mM Ca^{2+}) bubbled with 95% O_2 –5% CO_2 at 37°C. Once stable contractile function was achieved, the heart was arrested with modified isotonic Krebs solution containing 20 mM K^+ and 20 mM 2,3-butanedione monoxime. Passive LV function was characterized for 34 SHR and 35 WKY rats. The remaining hearts were processed for histological analysis. Plasma brain natriuretic peptide (BNP) levels were determined by radioimmunoassay (Endolab, Christchurch, New Zealand).

Characterization of passive LV function. LV pressure-volume relationships were characterized at each age end point (3, 12, 18, and 24 mo); numbers in each group were 8, 10, 9, and 8 for SHR, respectively, and 9, 9, 8, and 8 for WKY rats, respectively. With the heart attached to the Langendorff apparatus, a saline-filled balloon catheter was inserted into the LV via the mitral valve. The catheter was fixed by a purse-string suture in the left atrium and also attached to the Langendorff cannula. The balloon was fabricated from compliant polyvinylchloride film sealed around the end of a vinyl tube (2 mm OD) and stretched so that its undeformed volume was greater than that of the LV cavity. The balloon was inflated and deflated with a 1-ml glass syringe driven by a small stepper motor. Pressure was measured via a side port in the balloon catheter using a differential pressure transducer (Validyne Engineering) referenced to the level of the LV base. The volume displaced from the syringe was estimated from the position of the plunger. The experimental protocol was as follows: three cycles of balloon inflation and deflation between 0 mmHg and a specified maximum pressure at a steady rate of ± 0.036 ml/s. The maximum pressure was increased successively in the range 5–30 mmHg to minimize artifact due to strain softening and variable conformation of the balloon to the LV cavity surface. All data were

obtained for the third cycle of inflation to a maximum pressure of 30 mmHg. The stepper motor was controlled by software written in the LabVIEW programming language (National Instruments), which simultaneously acquired LV pressure and the change in LV volume. Data from 5 WKY rats and 6 SHR were excluded because of the onset of contracture or leakage of air into the system.

Histological analysis. Detailed histological analysis was carried out for two to five animal hearts at each age end point, making a total of 25 samples in total. While still on the Langendorff apparatus, the heart was perfusion-fixed with Bouin's solution and perfusion-stained for 2 h with picosirius red dye (0.1% sirius red F3BA in picric acid). The stained heart was left in Bouin's solution for 5–7 days; then 2-mm-thick equatorial rings were cut through the ventricles, and small transmural blocks were cut from the lateral LV free wall of these rings (35, 46). These tissue blocks were dehydrated in a graded ethanol series and then in propylene oxide, embedded in an agar resin (PROCURE 812, ProSciTech, Queensland, Australia), and polymerized for 48 h at 60°C.

Tissue blocks from these hearts were imaged using a purpose-built system that is described in detail elsewhere (35, 38). Briefly, the resin-embedded tissue block was mounted on a high-precision three-axis stage, which, together with a confocal laser scanning microscope, was used to acquire images at a series of focal planes from the surface of the block to a depth of 35 μ m. The surface was then milled to remove 30 μ m, and the process was repeated. Processing of the acquired images consisted of background correction, denoising using a wavelet denoising technique, and a Richardson-Lucy deconvolution to account for the finite extent of the point-spread function. Overlapping image subvolumes were assembled to form the complete 3D image, and histogram equalization was used to correct for attenuation of intensity with imaging depth. Tissue volumes were $\sim 4 \times 1 \times 0.3$ mm, with 1- μm^3 isometric voxels. Figure 1 shows an example block (Fig. 1A) and the subsequent steps of image analysis and quantitative measurement. Volume images were rendered and visualized using the freeware program Voxx (<http://www.indiana.edu/~voxx/>). Confocal images are presented in gray scale or in the "Glow" color palette, where black is low intensity (typically background), red is medium intensity (typically autofluorescence from the myocytes), and yellow/white is high intensity from the picosirius red (typically collagen, but also cell nuclei and other structures).

Total collagen volume fractions were estimated using a modified top-hat filter (37) on subblocks ($400 \times 400 \times 200 \mu\text{m}$) sampled from the middle of the heart wall and aligned with the laminar organization for comparisons between groups (Fig. 1B).

To compare structural components across the wall, it is helpful to create a transmural cross section that has a consistent orientation with respect to the local myocyte axis (39). The local myocyte orientation was computed at intervals along a transmural axis (perpendicular to the epicardium), defining the transmural fiber angle rotation (Fig. 1C). In this region of the LV free wall, myocytes are closely parallel to the epicardium, which allows for the definition of a curvilinear (and roughly helical) cutting plane perpendicular to the myocytes as they rotate along the transmural axis (Fig. 1D). This plane, which was flattened for viewing, shows a transmural section that is, by definition, transverse to the myocytes at all points across the wall, thereby displaying a microstructurally consistent view of the architecture (Fig. 1E). Various myocardial parameters, including myocyte area and specific measures of perimysial and endomysial collagen fraction, were measured on these flattened curvilinear images. Laminar width was measured at 200- μm intervals across the midwall (800 μm transmurally in total) and, by normalizing by the average myocyte diameter, is reported as the number of myocytes across each layer, which enables analysis of laminar remodeling independent of changes in cell size.

Higher-resolution images ($560 \times 560 \times 330 \mu\text{m}$, 0.4- μm^3 voxel) were also acquired from the midwall of one SHR and one WKY heart, both at 12 mo of age. Specific myocardial structural components

Fig. 1. Tissue imaging process and quantitative measurements of structure. *A*: entire imaged volume [12-mo-old spontaneously hypertensive rat (SHR)], $\sim 4 \times 1 \times 0.3$ mm at a voxel resolution of $1 \mu\text{m}^3$. *B*: 3-dimensional (3D) midwall block, extracted from the rotated square in *A* to align with laminae, which provides measurement of total collagen volume fraction. *C*: fiber angle measured transmurally shows a $\sim 180^\circ$ rotation from epicardium to endocardium. *D*: curvilinear 2-dimensional transmurular section defined perpendicular to myocyte fiber orientation. *E*: flattened image of *D* showing structurally consistent view of the transmurular section. Boxes show regions of uniform structure, in which myocyte area, laminar width, perimysial collagen, endomysial collagen, and capillary density are measured.

(myocytes, blood vessels, interlaminae spaces, and different collagen structures: endomysium, perimysium, and scarring) were segmented digitally on one of these higher-resolution images. Capillary density was measured by identifying, per myocyte, the number and distance of adjacent capillaries.

Measurements of type I collagen density were undertaken on a separate group of LV sections from 24-mo-old rats. Beating Langendorff-perfused hearts were arrested with cardioplegic solution. Transmurular segments were dissected from the LV free wall, fixed [4% paraformaldehyde-phosphate buffer solution (PBS), 2 days], and stored in PBS at 4°C . The tissue blocks were blotted and embedded in 6% agar with the epicardial surface face uppermost, and 120- μm sections were cut using a Vibratome (Campden). The sections were stained with F-actin and phalloidin-Alexa Fluor 488 diluted 1:25 in PBS for 3 h (Molecular Probes), washed with PBS, blocked for 1 h in 10% goat serum in PBS, and then immunostained overnight at 4°C with rabbit anti-rat type I collagen Ab diluted 1:20 in 5% goat serum-1% BSA-PBS (Chemicon, Temecula, CA). The slides were washed in PBS and then stained for 2 h with secondary Ab (Cy5 goat anti-rabbit Ab, 1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were washed (PBS), mounted (Citifluor, Agar Scientific), stored in darkness at 4°C , and imaged using a confocal microscope (TCS SP2, Leica). Five 5- μm optical sections ($\times 63$ magnification) of longitudinally orientated myocytes from each

heart were analyzed. Type I collagen area fraction was determined using a macro written in ImageJ to determine surface areas of labeled collagen. Collagen fraction is expressed as a percentage of total area.

Statistical data analysis. All data are expressed as group means $\pm$ SE, with the exception of the myocyte cross-sectional areas, which are presented as a box plot (see Fig. 5A). Data were analyzed using SAS/STAT software (SAS Institute, Cary, NC). A two-factor multivariate ANOVA with no repetition was used to test for age and disease effects on hemodynamic and morphological measurements, and between-group differences were assessed with protected *t*-tests with Bonferroni's adjustments (Table 1). All other analyses used a two-factor ANOVA with no repetition for strain, age, and strain-age interaction effects, with individual rats nested within strain-age. Statistical significance was based on $P \leq 0.05$.

RESULTS

Hemodynamic and morphological characteristics of SHRs and WKY rats throughout the study are summarized in Table 1. Compared with WKY rats, SHRs had elevated systolic blood pressure at all ages, but there was no significant difference in heart rate. Normalized heart and lung weights were consistently greater for SHRs than WKY rats, with marked increases

Table 1. Hemodynamic and morphological measurements

	SHR				WKY				Significance ($P < 0.05$)
	3 mo ($n = 12$)	12 mo ($n = 17$)	18 mo ($n = 11$)	24 mo ($n = 14$)	3 mo ($n = 12$)	12 mo ($n = 14$)	18 mo ($n = 11$)	24 mo ($n = 13$)	
BP, mmHg	155 $\pm$ 3	159 $\pm$ 5	172 $\pm$ 4	164 $\pm$ 6	109 $\pm$ 3	117 $\pm$ 3	111 $\pm$ 4	105 $\pm$ 2	S, S-A
HR, beats/min	380 $\pm$ 8	402 $\pm$ 8	429 $\pm$ 7	354 $\pm$ 24	332 $\pm$ 9	315 $\pm$ 9	383 $\pm$ 35	365 $\pm$ 13	A
Body wt, g	324 $\pm$ 6	421 $\pm$ 10	390 $\pm$ 13	420 $\pm$ 9	295 $\pm$ 5	415 $\pm$ 8	420 $\pm$ 5	429 $\pm$ 7	A, S-A
Heart wet wt/tibial length, g/cm	0.28 $\pm$ 0.01	0.36 $\pm$ 0.01	0.41 $\pm$ 0.02	0.54 $\pm$ 0.04	0.24 $\pm$ 0.00	0.29 $\pm$ 0.01	0.29 $\pm$ 0.01	0.31 $\pm$ 0.01	S, A, S-A
Lung wet wt/tibial length, g/cm	0.29 $\pm$ 0.01	0.31 $\pm$ 0.01	0.39 $\pm$ 0.02	0.50 $\pm$ 0.03	0.25 $\pm$ 0.00	0.27 $\pm$ 0.01	0.33 $\pm$ 0.02	0.34 $\pm$ 0.01	S, A, S-A

Values are means $\pm$ SE; n , number of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). S, strain; A, age; S-A, strain-age interaction.

in both groups between 18 and 24 mo, consistent with the onset of HF.

Echocardiographic measures of LV size and global systolic performance, together with plasma BNP levels, are presented in Fig. 2. LV end-diastolic diameter (Fig. 2A) was the same for both groups at 3 mo but increased more markedly with age for SHRs than WKY rats. Fractional shortening (Fig. 2B) was similar for both groups at 3 and 12 mo but was substantially reduced in SHRs between 18 and 24 mo. In both cases, there was a significant difference between strains and with age. Plasma BNP (Fig. 2C) was elevated in SHRs at all ages compared with WKY rats. In particular, BNP levels in SHRs increased dramatically from 18 mo but were not significantly affected by age in WKY rats.

Passive LV filling relationships for SHRs and WKY rats are compared in Fig. 3. There were qualitative differences between species in the LV pressure-volume curves (Fig. 3A). For SHRs, these are well to the left of WKY controls at 3, 12, and 18 mo, but there is a substantial shift to the right at 24 mo, presumably reflecting LV dilatation associated with HF. The variation in passive LV compliance with pressure is compared for species and age group in Fig. 3B. For filling pressures ≤ 10 mmHg, passive LV compliance was less at all end points for SHRs but varied considerably with age. LV compliance was markedly reduced at 12 mo and increased progressively thereafter. However, there was no difference in LV compliance for filling pressures >10 mmHg with age or species.

We have used the 3D imaging techniques (see MATERIALS AND METHODS) to demonstrate progressive changes in myocyte organization and collagen architecture with age (and disease progression) in SHRs.

Ordered laminar arrangement of myocytes was evident at all ages in WKY rats, and the separate components of perimysial collagen (35) could be identified. These include long cords within muscle layers, a meshwork of connective tissue surrounding muscle layers, and long fibers interconnecting adjacent layers. Corresponding perimysial collagen organization was observed in SHRs at 3 mo, but there was a striking change in the appearance of the perimysium from 12 mo. The perimysial collagen components surrounding muscle layers and those interconnecting layers fused, forming distinct sheets. Changes in endomysial collagen density also occurred with age in both groups but were most marked in SHRs. Representative transmural images at 3, 12, 18, and 24 mo for SHRs and WKY rats are shown in Fig. 4.

To quantify these morphological changes, perimysial and endomysial collagen fractions were estimated using a sampling grid (Fig. 1E) on flattened curvilinear planes extracted from the

transmural volume images. These provide a consistent viewing orientation at every point perpendicular to the myocyte axis (Fig. 1D). Finally, individual sections transverse to the myocyte were selected from each of the volume images acquired, and myocyte boundaries were manually digitized.

Variations in LV myocyte cross-sectional dimensions for SHRs and WKY rats are presented in Fig. 5A. Box plots were used for strain and age group comparison to avoid making assumptions about the underlying statistical distribution. At 3 mo, WKY and SHR myocyte cross-sectional areas were similar, and there was little variation in WKY rats with age. However, marked changes in myocyte cross-sectional dimensions were seen in SHRs between 3 and 12 mo, with increases in median and range. Median myocyte cross-sectional area remained elevated at 18 and 24 mo, and the range increased. Global collagen fraction was very similar for SHRs and WKY rats at 3 mo (Fig. 5B). However, there was a marked and relatively linear increase in global collagen fraction with age in SHRs, with a much less pronounced increase in WKY rats. Strain and strain-age interactions were statistically significant ($P < 0.0001$) for total collagen fraction.

Despite the evident fusion of the perimysial collagen at the interface between muscle layers from 12 mo in SHRs, no significant difference was found in transmural perimysial collagen fraction with strain or age (Fig. 5D). On the other hand, endomysial collagen density increased substantially in SHRs at 12 mo, and this was maintained at 18 and 24 mo. In WKY rats, endomysial collagen density was low at 3 and 12 mo but increased subsequently.

Changes in the number of myocytes per layer with age are compared for SHRs and WKY rats in Fig. 5C. WKY rats averaged three to four myocytes per layer, and this number did not change with age. Laminar architecture was similar in SHRs at 3 and 12 mo, but the number of myocytes per layer increased significantly at 18 and 24 mo. Strain and strain-age interactions for the number of myocytes per layer were statistically significant ($P < 0.0001$).

The results presented thus far indicate significant morphological changes in SHRs at ~ 12 mo. The 3D organization of myocytes and the endomysial and perimysial collagen scaffold were therefore imaged at higher resolution (0.4- μ m voxel dimension) in the midwall of SHRs and WKY rats at this age. Subvolumes from these higher-resolution images (200 $\times$ 200 $\times$ 50 μ m) are shown in Fig. 6 for both species with corresponding 3D images of the collagen (Fig. 6, C and D), and also in the accompanying movies (see supplemental movies in Supplemental Material for this article, available online at the Journal website), for which the collagen is shown by making

Fig. 2. Measures of left ventricular (LV) performance for SHRs (●) and Wistar-Kyoto (WKY) rats (○). *A* and *B*: echocardiographic estimates of LV end-diastolic diameter (EDD) and fractional shortening. *C*: plasma brain natriuretic peptide (BNP). Numbers of rats are shown in Table 1.

the lower-intensity tissue transparent. The tissue and collagen organization in these images reinforces many of the findings described above. The myocardium has a laminar arrangement in SHRs (Fig. 6, *A* and *C*) and WKY rats (Fig. 6, *B* and *D*), although myocyte hypertrophy, increased range of cross-sectional

dimensions, and increased numbers of myocytes per layer are evident in SHRs. The most striking differences are in the associated collagen framework. In the WKY specimen, the loose organization of the perimysial collagen cords connecting adjacent muscle layers has allowed these structures to separate during fixation and processing, whereas the perimysial collagen surrounding and interconnecting muscle layers is fused and thickened in the SHRs. In addition, the endomysial collagen surrounding individual myocytes is qualitatively thickened in the SHR segment and appears to have fused with the perimysial collagen cords within layers. The overall impression is that the connective tissue matrix is much more rigid in SHRs than WKY rats.

To quantify these differences, we have manually segmented one higher-resolution image from each strain. Myocyte and blood vessel boundaries, cleavage planes, and perimysial and endomysial collagen, as well as perivascular connective tissue and scar tissue, were accurately identified (Fig. 7), and the tissue fraction occupied by these structures is presented in Table 2.

As expected, the space occupied by cleavage planes is much greater in WKY rats than SHRs. All components are expressed as a fraction of the remaining (tissue) area, and the most striking difference between strains is the substantial increase in endomysial collagen in SHRs. While total perimysial collagen was similar in both strains, it was identified more readily at interlamina boundaries in SHRs, while in WKY rats the cross sections of the long perimysial cords could be seen within laminae. These cords were more difficult to distinguish in SHRs, as the proliferating endomysium encompassed the individual structures. Focal scarring, identified as large areas of replacement fibrosis, was evident in the SHR, but not the WKY, specimen. Finally, the proportion of vascular space to total tissue area is substantially reduced in SHRs relative to WKY rats.

The density of type I collagen was substantially greater in SHRs than in WKY controls at 24 mo (Fig. 8). This was due to increased expression of type I collagen in the thickened perimysial collagen between adjacent layers of myocytes (Fig. 8*A*) and also the focal scarring associated with replacement fibrosis (Fig. 8*C*). Overall, in the twelve 24-mo-old SHRs analyzed in this substudy, type I collagen density was more than twice as great as in the age-matched WKY controls (Fig. 8*E*).

DISCUSSION

The compensated response of the LV to pressure overload is concentric hypertrophy, with wall thickening that is associated with myocyte cross-sectional enlargement and increased LV passive stiffness (16). Eventually, this compensation manifests itself as diastolic failure, in which ejection fraction is preserved, but the capacity to increase cardiac output in exercise is compromised and pulmonary venous pressures are elevated, leading to congestion (16). Principal LV strains are maintained (33) or reduced (44), but LV torsion is increased (33, 44). The progression from compensated to decompensated HF in HHD is characterized by ongoing LV dilatation and reduction of ejection fraction. Characteristic changes in myocyte structure and the architecture of the cardiac ECM occur throughout this process.

The cardiac ECM defines the geometry of the ventricular chambers, and it provides the framework for organization of

Fig. 3. Passive LV filling relationships for SHRs (solid lines; $n = 8, 10, 9$, and 8 at $3, 12, 18$, and 24 mo, respectively) and WKY rats (dashed lines; $n = 9, 9, 8$, and 8 at $3, 12, 18$, and 24 mo, respectively, shown adjacent to the appropriate curve). A: LV pressure-volume curves. B: LV compliance (derived from A) as a function of filling pressure.

cardiac myocytes throughout the heart. It also contributes to mechanical coupling, transmitting forces within and across the heart wall, limiting the extension of myocytes during diastole, and storing energy during systole. These factors combine to produce highly effective mechanical performance in the normal heart. The LV undergoes large changes in shape and dimension throughout the cardiac cycle, and although there are substantial transmural gradients of 3D strain, myofiber extension is relatively uniform across the LV wall in systole and diastole. Experimental studies (25) and kinematic analyses (1, 26, 45) suggest that this depends on the following factors: the ellipsoidal geometry of the LV, the transmural rotation of myofibers, slippage or shearing of adjacent muscle layers, strain-locking mechanisms that prevent overextension of myocytes, and, finally, torsional deformations that occur throughout the cardiac cycle.

The perimysial collagen network plays an important role in meeting these functional requirements (35). In the normal heart, ventricular myocardium has a characteristic laminar architecture (24, 35). Layers of coupled myocytes three to five cells thick (myolaminae) are separated by cleavage planes but branch and interconnect with neighboring layers. We demonstrated previously (35) that the perimysial collagen network defines this 3D architecture and is composed of three distinct forms: 1) an extensive meshwork that surrounds layers of myocytes, 2) convoluted fibers connecting adjacent muscle layers, and 3) longitudinal cords within layers. Myolaminae provide structural units that enable local myocyte rearrangement or shearing to occur, while the dense array of longitudinal cords distributed throughout the LV myocardium limits passive extension along the myocyte axis. Finally, the 3D assembly of these connective tissue components accommodates myofiber

Fig. 4. Flattened transmural curvilinear images from SHRs and WKY rats at $3, 12, 18$, and 24 mo. Images are defined perpendicular to the transmurally rotating myocytes at all locations.

Fig. 5. **A:** box-and-whisker plot of myocyte cross-sectional area for SHRs (filled boxes) and WKY rats (open boxes). Box represents 1st quartile, median, and 3rd quartile; whiskers show 10th and 90th percentiles. Sample consisted of 50–150 myocytes. **B:** collagen fractions for SHRs (●) and WKY rats (○). Collagen fraction was measured at 5 locations. Values are means $\pm$ SE. **C:** myocytes per layer in midwall regions for SHRs (●) and WKY rats (○). Values are means $\pm$ SE; 20–70 layers were observed. **D:** average endomysial and perimysial collagen fractions for SHRs (filled symbols) and WKY rats (open symbols). Error bars represent data range; >1,000 observations. In all cases, observations are from 2–5 animals (samples) for each data point.

rotation and provides mechanical coupling across the ventricular wall.

Most previous studies of ECM remodeling in HHD have focused on global changes in collagen and other matrix components, as well as tissue factors that modulate collagen synthesis and turnover. The initial response of the ECM to sustained pressure overload is activation of transforming growth factor- β signaling pathways and reduced MMP-to-TIMP ratios (17, 41). This results in myocyte hypertrophy (parallel addition of sarcomeres) and progressive development of interstitial fibrosis, with elevated synthesis of type I collagen and increased cross-linking within the ECM (11). The increased LV passive stiffness that characterizes the compensated response to HHD reflects these changes. Less clear is the role of ECM remodeling in the LV dilatation and relative wall thinning that occurs during the progression to decompensated HF. It has been argued that LV dilatation occurs during this phase of HHD, because initial profibrotic processes are now reversed. That is, type I collagen is degraded and the extent of collagen cross-linking is reduced (3, 11). However, although the development of decompensated HF is associated with an elevation in MMP-to-TIMP ratios (18, 27) that precedes the development of dilatation (20), it takes place against a background of continuing interstitial collagen proliferation.

Few have sought to quantify, or even describe, the effects of HHD on different components of the collagen hierarchy. Rossi (36) reported a profound increase in endomysial collagen, thickening of perimysial collagen, and progressive disarray in postmortem human hearts where death was due to HHD. In contrast, López and co-workers (27) found marked reductions in perimysial and endomysial collagen in hypertensive patients with decompensated HF. This occurred in the presence of ongoing fibrosis, which they ascribed to increased deposition of perivascular and replacement collagen. Graham and Trafford (18) presented typical histological data that show a similar depletion in perimysial and endomysial collagen for ferrets in decompensated HF as a result of sustained aortic coarctation. However, overall collagen levels declined with progression of HHD, which was not observed elsewhere with pressure overload, either in animal disease models or clinical studies (17). The variability of these results reinforces the need to study HHD at standardized end points and to use animal disease models in which disease progression is comparable to human HHD. Finally, the 3D architecture of the cardiac ECM is explicitly considered in only one of these studies (36).

In this investigation, a novel extended-volume confocal microscopy technique (38) has been used to visualize the progression of structural remodeling in an established animal

Fig. 6. High-resolution ($0.4\text{-}\mu\text{m}^3$) images ($200 \times 200 \times 50\text{ }\mu\text{m}$) of LV midwall from 12-mo-old SHRs (A and C) and WKY rats (B and D). C and D: differences in collagen structures segmented from A and B, respectively. Collagen appears brighter, while myocytes have variable intensity.

model of genetic hypertension. LV myocyte arrangement and collagen architecture have been reconstructed in 3D and quantified in SHRs at four key stages in the progression of HHD. As far as we are aware, this is the first quantitative time-course analysis of such breadth in an animal model of HHD.

The SHR has been used extensively as an animal model of hypertension (4–6, 12, 21, 29, 32, 40, 43), because it replicates the classical progression of clinical HHD. The development of HHD in both strains is linked with activation of the renin-angiotensin system. Treatment with angiotensin-converting enzyme inhibitors in SHRs leads to regression of hypertrophy and fibrosis and normalization of LV stiffness (8–10, 31, 42), although the effectiveness of this treatment appears to be greatest earlier in the disease process (8–10, 31).

The temporal variation presented here for hemodynamic and gross morphological measurements (Table 1), measures of *in vivo* LV performance (Fig. 2), and passive LV filling relationships (Fig. 3) is consistent with previous findings for SHRs. However, this is the first presentation of such a comprehensive set of functional data over the full time course of disease development in this animal model. Systemic arterial pressure was elevated at 3 mo, with no apparent morphological or functional change. At 12 mo, there was clear evidence of diastolic dysfunction but no indication of symptomatic HF. There was also a striking reduction of passive LV compliance

(Fig. 3B), but fractional shortening was preserved and BNP levels were not increased with respect to those at 3 mo (Fig. 2, B and C). Systolic dysfunction was evident at 18 mo. The increase in lung wet weight (Table 1) is consistent with the development of pulmonary congestion, while LV dilatation, reduced fractional shortening, and increased BNP levels were seen (Fig. 2). Each of these measures was amplified with the progression toward end-stage HF between 18 and 24 mo. These data confirm that the end points selected for extended volume imaging and subsequent quantitative analysis of structural remodeling stratify the progression of HHD into four distinct stages: 1) early systemic hypertension, 2) diastolic dysfunction, 3) early systolic failure, and 3) decompensated HF.

A substantive new outcome of this work is that remodeling has been characterized systematically in SHRs throughout the progression from initial hypertension to decompensated HF. From 12 mo, myocyte cross section was increased, as has been shown previously (2), and was more variable (Fig. 5A). Laminar organization was less evident and less uniform than in controls at 18 and 24 mo, with increased numbers of myocytes per layer (Fig. 5C). Collagen deposition increased with age but was associated with striking changes in the 3D forms of the perimysial collagen network. The connective tissue meshwork at the surfaces of adjacent layers of myocytes and the convoluted fibers connecting these layers fused to form thickened

Fig. 7. Segmented high-resolution images from LV midwall from a 12-mo-old SHR (A and C) and a 12-mo-old WKY rat (B and D). Green, myocytes; yellow, blood vessels; dark blue, endomysium; red, perimysium; light blue, interlaminar space; gray, scarring.

planes of perimysial collagen at 12 mo (Figs. 4, 6, and 7). These planes were more dispersed and appeared less ordered, with the subsequent development of systolic HF at 18 and 24 mo. Despite this, there was no significant change in perimysial

collagen fraction with age in SHR (Fig. 5D). Type I collagen was substantially greater in SHR at 24 mo than in age-matched controls (Fig. 8). Finally, there was a marked proliferation of the endomysial collagen network from 12 mo (Figs. 5D, 6, and 7).

At first sight, the lack of change in perimysial collagen fraction appears to be at odds with the thickening of perimysial collagen evident in Figs. 4, 6, and 7 at 12 mo. For example, the perimysial collagen fraction measured in Fig. 7 and presented in Table 2 is substantially increased with respect to control. One might also have expected a reduction in perimysial collagen fraction thereafter because of the loss of laminar myocyte organization at 18 and 24 mo. However, the representative structural data in Fig. 5 demonstrate that these data are internally consistent. Myocyte cross section is enlarged at 12 mo (Fig. 5A), while the number of myocytes per layer increases from 18 mo (Fig. 5C); the combined effect is that the numbers of muscle layers per unit cross-sectional area are reduced progressively at 12 and 18 mo. Perimysial collagen fraction does not change, because it is offset by an ongoing thickening of the collagen between layers over the same period (Table 3). This trend is apparent in Figs. 4 and 7, where the number of muscle layers per unit area is less in SHR than in WKY controls at 12 mo and thereafter, but the collagen between

Table 2. *Quantified tissue components*

	SHR	WKY	Color
<i>Structure, %</i>			
Cleavage plane	1.1	18.8	Light blue
Myocardium	98.9	81.2	
Myocyte	67.4	82.0	Green
Endomysium	9.5	<2.0	Dark blue
Perimysium	11.1	6.9	Red
Scar	6.4	0.0	Gray
Vessel	5.5	10.8	Yellow
<i>Capillaries per myocyte CSA</i>			
Quartile			
25th	0.0025	0.0033	
50th	0.0036	0.0046	
75th	0.0049	0.0061	
100th	0.0120	0.0169	

Quantification of structures segmented from high-resolution SHR and WKY images and associated colors in Fig. 7, as well as distribution of capillaries associated with each myocyte, defined per unit myocyte cross-sectional area (CSA, μm^{-2}).

Fig. 8. Actin staining (green) and type I collagen-specific staining (red) from a 24-mo-old SHR (A and C) and a 24-mo-old WKY rat (B and D). Magnification $\times 63$. E: measured area fractions of type I collagen.

muscle layers is clearly thicker and brighter (more collagen). A corollary to this is that the increased perimysial collagen fraction presented in Table 2 is not representative. It reflects the spatial nonuniformity of structural remodeling in SHRs and underlines the potential risk of presenting individual image fields selected because they illustrate a characteristic feature of remodeling (in this case, thickening of perimysial collagen) as typical.

Our structural data are consistent with the findings of Rossi (36) for end-stage HF due to HHD but are at variance with those

presented by López et al. (27) for a similar patient cohort (see above). A point of difference is that Rossi, like us, visualized the cardiac collagen network in 3D across a relatively large LV transmural region and standardized the viewing plane in associated histological observations. On the other hand, López et al. studied much smaller microbiopsy specimens from the subendocardial layers of the interventricular septum, in which the capacity to control the orientation of histological sections was limited.

The structural remodeling in HHD alters myocyte structure and ventricular geometry but also fundamentally changes the

Table 3. *Perimysial collagen morphology changes*

	12 mo	18 mo	24 mo
SHR	↑	↑↑	↑↑
WKY	—	—	—

Qualitative changes in thickness of perimysial boundaries between layers with age for SHR and WKY rats relative to thickness at 3 mo. —, No change.

3D architecture of the cardiac ECM. It is evident that the fibrotic processes that occur early in HHD will stiffen the LV and lead to impaired diastolic function. However, our data do not support the view that the ventricular dilatation that accompanies the subsequent development of decompensated HF is caused by reversal of profibrotic processes or “softening” of the cardiac ECM. Although LV compliance for SHRs increased progressively at 18 and 24 mo compared with 12 mo, it was substantially less than that for age-matched WKY controls at LV filling pressures <10 mmHg and did not change significantly with age at filling pressures ≥10 mmHg (Fig. 3B). Furthermore, we found that type I collagen in the perimysial network increased, rather than decreased, at 24 mo (Fig. 8) and note that increased collagen cross-linking has been reported for patients with systolic HF secondary to HHD (28). Therefore, the elevated MMP-to-TIMP ratios that characterize the progression to decompensated HF in HHD (17, 30, 34) most likely reflect ongoing reorganization of the ECM scaffold, rather than its dissolution.

Structural remodeling at a range of scales may impact on systolic function in HHD. At the cell level, derangement of the cytoskeleton and sarcoplasmic reticulum is thought to contribute to altered Ca^{2+} homeostasis and apoptosis (13, 22). It is also argued that hypertrophy reduces the effectiveness of diffusive oxygen delivery to cardiac myocytes, leading to cell death. The number of capillaries per myocyte increased for SHRs from 12 mo, consistent with ECM-mediated angiogenesis through cellular signaling pathways such as fibroblast secretion of vascular endothelial growth factor (7). However, the number of capillaries per unit cell cross-sectional area was reduced, and the distribution was skewed toward lower densities compared with control (Table 2). A greater population of cells was therefore at risk of impaired oxygen delivery in SHRs. On the other hand, the main changes in myocyte cross section occurred by 12 mo, well ahead of the progression to decompensated HF.

At the tissue level, the remodeling of the cardiac ECM reported here must affect regional ventricular stress and deformation during systole. Furthermore, altered mechanical loading itself probably influences the process of remodeling. The perimysial collagen network provides the scaffold that organizes the ventricular myocardium into groups or layers of myocytes; it allows for reorganization of muscle layers, limits myocyte extension, and transmits forces across the ventricular wall. It follows that these mechanical functions will be affected by the wholesale change in the forms of the 3D perimysial collagen network and the ongoing disruption of the normal laminar arrangement of myocytes that occurs with the progression of HHD. For instance, it is thought that shearing or slippage between adjacent layers of myocytes contributes to effective LV ejection by enabling greater systolic wall thickening than would otherwise be possible (1, 25). Scarring of the

perimysial collagen between muscle layers would therefore be expected to decrease the extent to which wall thickness can change throughout the cardiac cycle. Ejection fraction is likely preserved initially in HHD, because reduced LV wall thickening is offset by increased torsional deformation (33, 44), also a result of this structural remodeling. However, we hypothesize that ongoing remodeling of the 3D perimysial collagen network and increasing disorder in the laminar arrangement of the myocardium contribute to the decline in ejection fraction during the progression to decompensated HF.

Limitations. The fact that an animal disease model, rather than human HHD, has been studied may be viewed as a limitation. The SHR replicates key features of the progression to decompensated HF in HHD that occurs in many patients, while the effectiveness of treatment with angiotensin-converting enzyme inhibitors is similar in both (5). However, the notion that there is a single pathway to HF in HHD appears to be at odds with a significant body of experimental and clinical data (14). A further limitation is that we have not assayed tissue factors associated with synthesis and turnover of collagen in our time-course study. However, data for MMP2 and TIMP4 have been presented for SHRs and age-matched WKY controls (30) at comparable end points. Finally, the size and resolution of the pediatric echocardiographic probe used to determine cardiac dimensions and fractional shortening probably introduced greater variability in these data than would have been the case had a specialized rodent probe been used. Nevertheless, significant changes were detected, and these were consistent with independent measures of systolic and diastolic function. We have also not addressed whether the origin of the structural changes is a result of the hypertension or a combination of other factors. All these issues are clearly relevant and should be the subject of further studies.

Perspectives. The structural changes quantified in this animal model of HHD are more complex than has previously been proposed. The development of diastolic dysfunction is associated with extensive remodeling of the 3D collagen framework; the perimysial collagen between adjacent muscle layers becomes fused and thickens, while there is a marked proliferation of the endocardial collagen network that surrounds individual myocytes. The LV dilatation that accompanies development of decompensated HF is not driven by dissolution of the endomyocardial and perimysial networks or degradation of type I collagen. Instead, it is associated with ongoing remodeling of the collagen scaffold that occurs against a background of increasing interstitial fibrosis. The laminar arrangement of myocytes becomes disrupted and increasingly disordered. The perimysial collagen between layers continues to thicken but is more sparsely and less uniformly distributed. As a result, the perimysial collagen fraction remains unchanged. Because the cardiac ECM is a complex 3D structure, these changes are most reliably quantified using appropriate volume images and across regions that are sufficiently large to account for inherent spatial nonuniformity. Improved theoretical biomechanics models that incorporate key features of ECM structure are needed to better understand the mechanisms by which structural remodeling affects cardiac function. We believe that the present study is a step toward this end.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

I.J.L. and B.H.S. are responsible for conception and design of the research; I.J.L., A.J.P., G.B.S., and B.H.S. analyzed the data; I.J.L., A.J.P., G.B.S., G.W., R.N.D., and B.H.S. interpreted the results of the experiments; I.J.L., A.J.P., and G.B.S. prepared the figures; I.J.L., A.J.P., G.B.S., and B.H.S. drafted the manuscript; I.J.L., G.B.S., and B.H.S. edited and revised the manuscript; I.J.L., G.B.S., and B.H.S. approved the final version of the manuscript; A.J.P., G.W., and R.N.D. performed the experiments.

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