

Hydroxyproline and Passive Stiffness of Pressure-induced Hypertrophied Kitten Myocardium

JOHN F. WILLIAMS, JR., RALPH D. POTTER, DANNY L. HERN, BABU MATHEW, and
WILLIAM P. DEISS, JR., *Department of Medicine, The University of Texas
Medical Branch, Galveston, Texas 77550*

ABSTRACT Passive stiffness and hydroxyproline content of myocardium hypertrophied by pressure-loading were determined in kittens 2, 8–16, and 24–52 wk after pulmonary artery banding, which initially elevated right ventricular systolic pressure by 10–15 mm Hg. Right ventricular mass increased by ~75%, three-quarters of which occurred during the first 2 wk after banding. Passive stiffness was assessed from resting length-tension relations of isometrically contracting isolated right ventricular papillary muscles. Stiffness constants, α and β were determined from the relationship $\sigma = \alpha(e^{\beta\epsilon} - 1)$ where σ = stress and ϵ = Lagrangian strain. Elastic stiffness ($d\sigma/d\epsilon$) was derived from: $d\sigma/d\epsilon = \beta\sigma + \beta\alpha$. Right ventricular hydroxyproline increased in proportion to muscle mass so that hydroxyproline concentration remained unchanged after banding. Both α , β , and elastic stiffness-stress relations were similar to values in nonbanded controls. Thus, we did not observe an increase in passive stiffness or hydroxyproline concentration of pressure-induced hypertrophied myocardium in contrast to most previous studies.

INTRODUCTION

An increase in passive stiffness of pressure-induced hypertrophied myocardium in the experimental animal has been observed by several investigators (1–3) including ourselves (4). Furthermore, the increase in passive stiffness would appear to be due at least in part to a concomitant increase in collagen that accompanies the hypertrophic process (3, 5–7). However, it is unclear whether these changes represent a fundamental characteristic of pressure-induced hypertrophy or a peculiarity of the experimental model. The observa-

tion of Bishop and Melsen (8) that pulmonary artery banding in the cat produced myocardial necrosis and fibrosis, whereas neither was found in cats with congenital pulmonary valve stenosis, supports the latter possibility. Furthermore, it is clear that collagen concentration can increase, decrease, or remain unchanged during development of hypertrophy depending on the stimulus for increased growth (9) and that an inverse relationship between connective tissue and passive stiffness can be found under certain conditions (10). Therefore, we attempted to develop a model of pressure-induced hypertrophy in which hypertrophy developed more gradually than in the usual animal models and compared passive stiffness and the hydroxyproline response in these hearts.

METHODS

Growing kittens 12–20 wk of age were anesthetized with intraperitoneal sodium pentobarbital (35 mg/kg) following which the chest was opened and the main pulmonary artery isolated. Right ventricular pressure was measured by direct 25-gauge needle puncture of the free wall and a band of sufficient size to elevate right ventricular systolic pressure by 10–15 mm Hg was secured around the main pulmonary artery. The band was composed of silastic tubing through which passed a copper wire of desired length and a silk suture. The suture was then tied to maintain band size and was also secured to fibrous tissue in the region.

The animals were killed 2, 8–16, or 24–52 wk after banding. The hearts were removed and the thinnest right ventricular papillary muscle dissected free and placed in a myograph containing modified Krebs's solution of the following concentrations (mM): Na^+ , 144; K^+ , 4.0; Ca^{++} , 2.5; Mg^{++} , 0.5; H_2PO_4 , 1.0; HCO_3^- , 25; Cl^- , 128, and glucose, 5.6. The solution was maintained at a temperature of 30°C and was bubbled vigorously with 95% O_2 –5% CO_2 , which produced a pH of 7.4 and a PO_2 exceeding 500 mm Hg. The nontensioned end of the muscle was held rigidly by a plastic clip attached to a short metal rod that passed through the bottom of the myograph and was connected to a Satham force transducer (Satham Instruments, Inc., Oxnard, Calif., model GI-

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4-250). The tendinous end was secured by a short silk suture to the long arm (10:1 ratio) of a lever attached to a displacement transducer (Schaevitz Engineering, Camden, N. J., model R4BS), which in turn was secured to a rigid stand. The compliance of the system without muscle was 2 $\mu\text{m/g}$ and the equivalent mass of the lever system was 150 mg. Micrometers appropriately placed above the lever were used to obtain isometric contractions at various muscle lengths or isotonic contractions with light preloads. The muscle was stimulated with square wave impulses of 4-5 ms duration at a frequency of 12/min and voltage 10% above threshold using field electrodes parallel to the long axis of the muscle.

After the muscle had contracted isotonically with a light preload for 45-60 min, maximal velocity of shortening was measured. Isometric contractions then were produced and length-tension relations determined after 0.1-mm increments in length from zero resting stress to the point at which active force first declined from its maximum. Force measurements were made only after stress relaxation was complete. Three length-tension curves were obtained for each muscle. Muscle length at peak active force development (L_{max})¹ was measured with a calibrated reticle and cross sectional area determined from wet weight and length at L_{max} assuming the muscle to be a cylinder with a specific gravity of 1.0.

Active muscle performance was assessed from measurements of maximal measured velocity of isotonic muscle shortening with a light preload and from active force development at L_{max} . We assessed passive stiffness from the stress-strain relationship calculating the elastic constants α and β as described by Glantz and Kernoff (11). Because the stress-strain relationship of cardiac muscle is monoexponential over the physiologic range of stress (11, 12), the elastic constants can be derived from: $\sigma = \alpha (e^{\beta\epsilon} - 1)$ where σ = force/instantaneous cross sectional area and ϵ (Lagrangian strain) = $1 - l_0/l$, l_0 being unstressed muscle length. We also calculated the tangent modulus or elastic stiffness ($d\sigma/d\epsilon$) from $d\sigma/d\epsilon = \beta\sigma + \beta\alpha$ (11). Since the stress-strain relationship is exponential, elastic stiffness is linearly related to stress. The theory pertaining to and derivation of these expressions has been described by Glantz (13) and the importance of the elastic stiffness-stress relationship has been discussed by Mirsky (2).

The right ventricle was dissected from the left ventricle plus the septum and the weight of each specimen obtained. Fat was trimmed from the ventricles, which were cut into small pieces, placed in flasks, and extracted with acetone at 4°C using a continuous shaker. Three changes of acetone were made at 24-h intervals. The acetone-extracted tissues were freeze-dried, ground to pass a 20-mesh screen, and maintained *in vacuo* over phosphorous pentoxide for at least 16 h.

Hydroxyproline was measured using a modification of method "A" of Bergman and Loxley (14). Appropriate aliquots (1-9 mg) of the dried tissue were weighed, 2 ml 6 N HCl added for hydrolysis and the tubes maintained at 105°C for 16 h. The samples were then decolorized with humin precipitant, filtered through Whatman No. 1 filter paper (Whatman, Inc., Clifton, N. J.), and the filtrates taken to dryness using air jets. The samples were made to a convenient volume with H₂O, 1-ml aliquots taken for assay and

¹ Abbreviations used in this paper: L_{max} , muscle length at peak active force development; RV/LV, right ventricular, left ventricular weight ratio.

2-ml i-propranolol added. 1 ml of chloramine-T oxidant solution and after 4 min 2 ml of Ehrlich's reagent were then added. Color was developed at 60°C for 21 min. The samples were allowed to remain at room temperature for 1 h after which the optical density was determined at 562 nm. Hydroxyproline standards ranging in concentration from 1 to 6 $\mu\text{g/ml}$ H₂O were run along with the samples. Final sample concentrations were determined using a linear curve constructed from the standards' optical density readings. All hydroxyproline data were calculated on a dry weight basis. In selected hearts hydroxyproline was measured in papillary muscles.

Similar studies were performed in nonbanded kittens 12-20 and 52 wk of age.

Statistical analyses were performed using analysis of variance (15) unless otherwise stated.

RESULTS

Resting tension at any given length was found to be significantly higher in the first length-tension curve than in the succeeding two in both hypertrophied and nonhypertrophied muscles. Since no systematic differences existed between the second and third length-tension curves, the first was discarded and the latter two averaged.

Anatomic data, mechanical properties, and elastic constants for all groups are presented in Table I. Right ventricular-left ventricular weight ratio (RV/LV), active mechanical properties and the passive elastic constants of the nonbanded animals studied at 12-20 or 52 wk are quite comparable. Thus, for statistical analysis the data from these two groups was combined.

Banding resulted in the rather rapid development of right ventricular hypertrophy that occurred principally within the first 2 wk after banding with only a small further increment during the remainder of the study. Ultimately, RV/LV increased by an average of 75% with three-quarters of this increase occurring during the first 2 wk. Both peak active force and velocity of shortening declined 2 wk after banding but had returned to control values by the 8-16-wk study period. Neither the α - or β -elastic constants of hypertrophied muscles were significantly different from that of nonbanded animals.

Elastic stiffness-stress relations are depicted in Fig. 1. Although elastic stiffness of nonhypertrophied muscles exceeded that of each of the hypertrophied groups over the entire stress range, the differences among groups are not significant.

The hydroxyproline content in milligrams per ventricle is given in Fig. 2. As expected, total hydroxyproline in nonbanded animals was greater in the left ventricle than the right ventricle and both increased as the animals grew and heart weight increased. Total hydroxyproline in the left ventricle of banded animals also increased, but never significantly exceeded the

TABLE I
Anatomic and Functional Data for All Groups, Values = Mean±SEM

	n	RV/LV	Cross-sectional area at Lmax mm ²	Peak force g/mm ²	Max. velocity shortening ML/s	β	α g/mm ²
Controls							
12-20 wk (C1)	10	0.29±0.02	1.1±0.2	5.0±0.4	1.01±0.09	16.1±2.0	0.10±0.02
52 wk (C2)	11	0.31±0.01	1.3±0.1	5.1±0.6	0.90±0.12	16.1±1.3	0.07±0.01
Banded							
2 wk	5	0.47±0.05	1.4±0.2	3.0±0.3	0.70±0.18	15.2±1.9	0.11±0.02
8-16 wk	11	0.49±0.03	1.0±0.3	4.9±0.5	0.95±0.06	14.3±1.6	0.09±0.02
24-52 wk	13	0.46±0.02	1.3±0.2	5.6±0.5	1.20±0.15	13.8±2.0	0.10±0.01

ML/s, muscle lengths/s.

values found in nonbanded animals. However, right ventricular hydroxyproline rapidly and continuously increased after banding eventually reaching values about twice that of nonbanded animals of comparable age ($P < 0.001$).

Hydroxyproline concentrations in milligrams per 100 mg of ventricle are presented in Fig. 3. As reported by others in nonbanded cats (5) right ventricular hydroxyproline concentration exceeded that of the left ventricle. Although the average right and left ven-

tricular concentrations in the older animals were less than their respective values in the younger nonbanded animals, the differences are not significant statistically. In banded animals both right and left ventricular hydroxyproline concentrations were comparable to their respective values in nonbanded animals throughout the study period.

We determined passive stiffness in papillary muscles and hydroxyproline in the right ventricular free wall of all animals. However, we also measured hydroxyproline in the papillary muscles of selected banded and nonbanded animals. Comparison of these measurements to those in the right ventricular free wall and the passive elastic constants in these animals is given in Table II. By paired comparison *t* testing hydroxyproline concentration in the papillary muscles was significantly less than that in the right ventricular

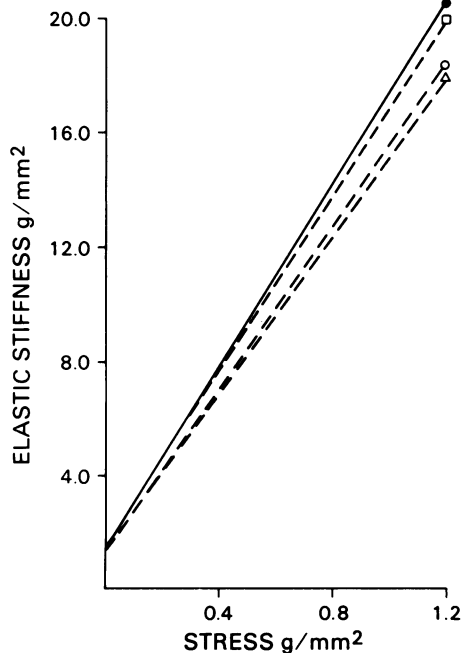


FIGURE 1 Elastic stiffness-stress relations in nonbanded (—●) 2-wk banded (---□), 8-16-wk banded (---○), and 24-52-wk banded (---△) animals.

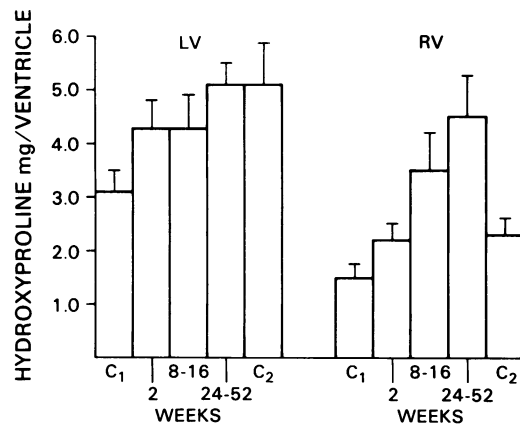


FIGURE 2 Total hydroxyproline in the left ventricle (LV) and right ventricle (RV) of various groups. C₁ and C₂ represent nonbanded animals 12-20 and 52 wk of age, respectively. 2 wk, 8-16 wk, and 24-52 wk refer to weeks after banding.

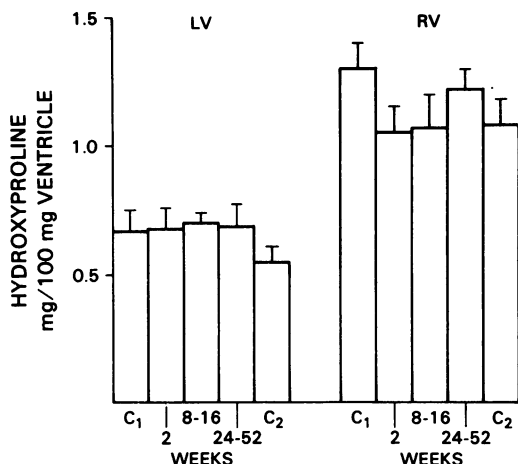


FIGURE 3 Hydroxyproline concentration in mg/100 mg dry wt in various groups as identified in Fig. 2.

free wall in both hypertrophied and nonhypertrophied muscles. However, as with right ventricular free wall hydroxyproline concentration there were no significant differences in papillary muscle hydroxyproline concentrations among banded groups. Similarly, elastic constants among groups were comparable.

DISCUSSION

The unchanged passive stiffness of hypertrophied myocardium in this study is in contrast to previous reports (1-3) including our own (4). Furthermore, the constant hydroxyproline concentration as hypertrophy developed also is opposite that observed by others in pressure-induced cardiac hypertrophy (3, 5, 6). Comparison of this and previous studies revealed that these differences could not be attributed to differences in species studied (2, 4, 5) or extent of hypertrophy

(1, 2, 4). We did use growing kittens in which the initial ventricular load produced by banding was slight in comparison to previous studies which used adults with greater degrees of banding. Thus, our differences could be attributed to either a difference in the response of kitten and adult myocardium to banding or to differences in the initial loading conditions.

A major objective of this study was to develop a feline model in which increased right ventricular afterload occurred gradually. We hypothesized that if a minimal fixed pulmonary artery constriction was produced in kittens, the resistance to ejection would gradually increase as the animal grew and cardiac output rose. Others previously had used a similar method to successfully produce slowly developing left ventricular hypertrophy in dogs (16). Unfortunately, this did not occur and hypertrophy developed fairly rapidly, the principal increase occurring during the first 2 wk after banding with only a slight further increment thereafter. At 2 wk dense fibrosis was found around the band and we suspect that an inflammatory reaction reduced pulmonary artery diameter more quickly than anticipated. Nevertheless, both the load and hypertrophy occurred more slowly in this model than in a banded adult cat model used previously in our laboratory (17). We examined seven additional kittens 1 wk after banding in a similar manner and found the RV systolic pressure averaged 40 ± 4.0 mm Hg and the RV/LV ratio 0.38 ± 0.03 , whereas in six adult cats 1 wk after banding in the more standard manner (17) RV systolic pressure and RV/LV averaged 48 ± 4 mm Hg and 0.49 ± 0.02 , respectively, both values being significantly greater ($P < 0.05$) than their respective values in kittens banded for 1 wk. That the myocardium responds differently to rapidly or slowly imposed loads is evident from the observations of Bishop and Melsen (8) who found right ventricular myocardial necrosis

TABLE II
RV Free Wall and Papillary Muscle Hydroxyproline (HP) Concentration and Elastic Constants Each Determined in the Same Animal

	n	HP concentration		Elastic constants	
		RV	PM	β	α
		mg/100 mg		g/mm ²	
Nonbanded					
12-20 wk	4	1.3 ± 0.1	1.0 ± 0.2	15.4 ± 1.4	0.09 ± 0.01
52 wk	4	1.2 ± 0.2	1.0 ± 0.1	16.2 ± 1.1	0.07 ± 0.01
Banded					
8-16 wk	5	1.6 ± 0.2	0.9 ± 0.1	15.5 ± 0.9	0.10 ± 0.02
24-52 wk	4	1.5 ± 0.2	1.1 ± 0.1	14.2 ± 1.3	0.09 ± 0.01

Values, mean \pm SEM.

PM, papillary muscle.

and fibrosis in cats after pulmonary artery banding, but not in cats with right ventricular hypertrophy from congenital pulmonary valve stenosis. Others who have reported increased passive stiffness (1, 2) increased collagen concentrations (5-7) or both (3) in pressure-induced hypertrophied myocardium have used models more analogous to our adult model in which we also found passive stiffness to be increased (4).

If the rapidity with which hypertrophy develops is a reflection of the magnitude of the initial load that, in turn, determines the extent of myocardial necrosis and fibrosis, then our kittens should have a lesser degree of fibrosis than adult models. This would account for the differences in both stiffness and hydroxyproline concentrations between these models. Unfortunately, we do not have histologic confirmation of our hypothesis but others have observed a strong correlation between collagen concentration and histologic estimation of connective tissue (18). The study of Peterson et al. (19) demonstrating normal stiffness of hypertrophied myocardium in patients with aortic stenosis, the preliminary observations of Serizawa et al. (20) of normal stiffness in an experimental model of slowly developing pressure-induced hypertrophy and unchanged collagen concentration under the latter conditions (18, 21) support our hypothesis regarding the interrelationship of loading conditions, the rapidity with which hypertrophy develops, myocardial fibrosis, and stiffness.

Although we cannot exclude the possibility that kitten myocardium responds differently than adult myocardium to pressure loads, kittens of this age (12-20 wk) at the time of banding are similar to adults in terms of the RV/LV ratio and mechanical performance including passive stiffness. The observation of others (22) that kitten ventricular myocardium is less stiff than adult myocardium was made in kittens much younger than those of our study and an increase in passive stiffness with age in cats has not been a universal finding (23).

The cross-sectional area of our papillary muscles was larger than optimum and some degree of core hypoxia may have existed. However, there were no significant differences in the cross-sectional area among groups and results should be comparable. Limiting the study to hypertrophied muscles of optimal cross-sectional area, i.e., $<1.0 \text{ mm}^2$, would have necessitated the exclusion of 60% of our muscles. Because of the inordinate costs involved we utilized muscles up to 2.0 mm^2 that still required us to exclude 30%.

The hydroxyproline concentration of our papillary muscles was significantly less than that of the right ventricular free wall in both our banded and non-banded animals by paired comparison *t* testing. To our knowledge, no such comparisons have been made pre-

viously. Others have reported that hydroxyproline concentration of left ventricular papillary muscles was comparable to that of the free wall in normal rats, whereas hydroxyproline concentration of the papillary muscles was less than that of the free wall in spontaneously hypertensive animals (24). Importantly, as in the free wall, there were no significant differences in hydroxyproline concentration among our nonhypertrophied and hypertrophied papillary muscles and thus our conclusions remain valid. Nevertheless, caution should be exercised in extrapolating data derived from papillary muscles to that of the entire ventricle. The papillary muscle does participate in the hypertrophic process that occurs after pulmonary artery banding in the cat as demonstrated by others (25).

Several methods for assessing passive stiffness have been proposed but it is unclear as to which is the most valid (26, 27). A principal argument against the use of stress-strain relations using either natural or Lagrangian strain has been the necessity for determining unstressed muscle length. Certainly, at low levels of stress small increments in stress are associated with large increments in muscle length and accurate measurements of this relationship are difficult. However, we found that with high sensitivity tracings, measurements of muscle length at the point where resting stress first rises above zero are reproducible, i.e., $\pm 8\%$. Furthermore, Glantz and Kernoff (11) have shown that β is relatively insensitive to small changes in initial muscle length.

The anatomic determinants of passive stiffness have not been elucidated completely although there is considerable evidence that the connective tissue matrix and its collagen component play a major role (3, 28). Many authors have measured collagen as hydroxyproline since traditionally it has been believed that hydroxyproline formed a constant fraction of mammalian collagen (29). However, this is not invariably so and the fraction of collagen composed of hydroxyproline may vary according to the type collagen (30). We cannot exclude the possibility that collagen type changed in our hypertrophied hearts as occurs in other tissues in response to injury (31, 32). If such changes did occur they were not sufficient to alter passive stiffness.

In conclusion, we observed unchanged passive stiffness and hydroxyproline concentration in hypertrophied myocardium of pulmonary artery banded kittens indicating that neither increased stiffness or collagen concentration is a universal characteristic of hearts hypertrophied by pressure loading. Our differing results appear to be due to the use of a younger animal or to the less abrupt imposition of the load. Although we favor the latter explanation the data supporting this belief currently is inconclusive.

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