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SHIFTS IN CONTRACTILE REGULATORY PROTEIN SUBUNITS TROPONIN T AND TROPONIN I IN CARDIAC HYPERTROPHY

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SUMMARY : To examine the molecular basis of hypertrophied heart failure, we investigated the changes in cardiac contractile regulatory proteins. The guinea pigs were subjected to chronic pressure overload with aortic banding to induce ventricular hypertrophy, and in-situ pressure-volume relations were recorded together with biochemical characterizations to ascertain the contractile modifications. Immunoblots of left and right ventricular samples revealed four distinct troponin T isoforms, which underwent alterations during hypertrophy. The higher molecular weight bands TnT1 and TnT2 shifted towards the lower molecular weight isoforms TnT3 and TnT4. For TnI, a single prominent band was detected, whose intensity also increased with pump failure. The findings provide the first direct evidence of TnT and TnI shifts in an experimentally induced hypertrophied heart failure and has novel mechanistic implications for the future studies.

Studies of the mechanisms underlying myocardial hypertrophy remain the focus of intense multidisciplinary research, but the precise molecular basis of this remains obscure. Maintenance of normal myocardial contractility depends on both the adequate delivery of activator calcium to the myofilaments as well as on the contractile responsiveness of the myofilaments to it (1,2). A great deal has been learned regarding modulations in the free calcium transients of hypertrophied myocardium (3-7), but how the myofilament contractility is modified remains unsettled. The present study concerns with biochemical and structural characterizations of the myofilament regulatory proteins, esp. TnT and TnI, accompanying myocardial hypertrophy and heart failure in a well defined and reproducible experimental model.

The results indicate significant TnT and TnI alterations, which provides the first strong evidence that regulatory protein changes accompany myocardial hypertrophy in a reproducibly induced heart failure. The findings also suggest novel avenues for future explorations into the molecular basis of the underlying alterations.

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METHODS

Aortic Banding: A minimally-constricting loop of Tygon tubing was fixed around the ascending aortas of young (225-275 gram) male guinea pigs as described previously (8). This leads to a gradual increase in left ventricular overload and development of marked left ventricular hypertrophy as the animals grow. Additionally, aortic banded guinea pigs eventually (2-4 months postoperatively) also develop clinical dyspnea, which is uniformly associated with marked pulmonary congestion and right ventricular hypertrophy. All of the aortic banded guinea pigs in this study had these manifestations.

In-Situ Left Ventricular Pressure and Volume Measurements: Guinea pigs were anesthetized (sodium pentobarbital 50 mg/kg) and ventilated (Harvard rodent ventilator) prior to instrumentation of the left ventricle in-situ with a Millar (model SPR-249, Millar Industries) catheter inserted through the apex, and ultrasonic crystals placed to measure the LV maximum and minimum diameters using a sonomicrometer (Triton Industries). Standard left ventricular pressure-volume loops (Figure 1) were generated by a series of vena cava occlusions (9) and data were analyzed using custom made software. Left ventricular chamber volume was determined as: $LVCV=(3.14/6)(AxB^2)$ - LVM, where LVCV is left ventricular chamber volume in ml, A and B are the lengths of the major and minor axes in cm, and LVM is left ventricular mass in grams. Stress-strain relations were derived from these data using a spherical model as described by Glantz et al. (10). Postmortem hearts were trimmed of the atria and great vessels, the RV free wall dissected, and the RV free wall and the LV were blotted and weighed.

Figure 1A is an example of a series of pressure-volume loops obtained in-situ for a normal guinea pig left ventricle during a series of vena cava occlusions. The upper left and lower right borders of the "envelope" formed by these loops represent the systolic and diastolic pressure-volume relations, respectively. Subtraction of the volume attributable to the left ventricular myocardium (left ventricular mass divided by specific gravity) produces systolic and diastolic pressure-volume relations such as those shown in Figure 1B for a normal (circles) and a hypertrophied failing (triangles) heart. Ventricular pressure was then converted into myocardial wall stress by adjusting for chamber diameter and myocardial wall thickness resulting in the plots shown in Figure 1C for the same two hearts. Extrapolation of the diastolic stress-strain relations to zero stress provides a standardized estimate of chamber circumference. Lastly, to provide measures of intrinsic myocardial active stress and intrinsic myocardial wall stiffness, the ordinate (strain, in cm) was converted to Lagrangian strain (change in circumference) by normalizing to chamber circumference at zero stress, and determining the slopes of the systolic and diastolic relations, respectively (Figure 1D).

SDS-PAGE and Western Immunoblots: Protein electrophoresis of the ventricular strips was carried out on 8% polyacrylamide discontinuous Laemmli gels (16 x 20 cm) using the BioRad Protean II slab cell. Tissue samples (approximately 3 mm long and 150 μm thick specimens) in 60 μl SDS-sample buffer were vigorously dissolved using microtip ultrasonication (Bronson Sonifer, Model 200). The electrophoresis was carried out under constant-current conditions as before (11). Two gels were run concurrently and the same sample (60 μl) was divided evenly between the paired gels. After the run, one gel of the pair was silver-stained (see below) for estimation of the relative contents in the various bands and the second of the pair was used for immunoblotting.

Silver-staining was carried out using a modified BioRad procedure (Silver Stain Kit, BioRad) with the additional step of equilibrating with 5 % glutaraldehyde for fixation. These procedures as well as the identification of the various bands were done as previously described (11,12), and the identification was cross-checked with immunoblots wherever appropriate.

For Western blots, the paired unstained gel was transblotted onto a nitrocellulose membrane (0.45 μm pore size; Scleicher & Schuell Co.) using the BioRad Trans Blot Cell. The membrane was probed with antibodies using the ECL Detection Kit (RPN 2106, Amersham).

The monoclonal antibodies to both troponin I (gift of Dr. S. Schiaffino, Padova, Italy) and to troponin T (JLT-12, Sigma Immunochemicals) were used. The emergent bands were analyzed by scanning (Hewlett-Packard Scanjet II, 8 bits, 300 dpi, 256 gray scale), and computing the area and mean pixel density of each band using image processing software (Imagepro Plus 2.0, Media Cybernetics).

Statistical Analysis: Comparisons between the two groups on body and tissue mass were done by Student's t-test. Comparisons of derived coefficients and intercepts (from stress-strain analysis), which cannot be assumed to have Gaussian distribution, were made by the Wilcoxon Mann-Whitney test (13). Effects of the aortic banding procedure and ventricular side (right versus left) on troponin T isoform distribution were evaluated by analysis of variance (ANOVA). The data are expressed as means $\pm$ S.D, wherever appropriate.

RESULTS

The guinea pig aortic banding model was developed specifically to provide a means of studying heart failure which develops gradually and reproducibly and whose etiology is well defined. Systolic and diastolic modifications were verified by the stress-strain analyses. Immunoblots of TnT and I were investigated to develop insights into the molecular changes underlying myocardial hypertrophy.

The banded pigs were allowed to mature until clinical signs of heart failure were evident. Marked biventricular hypertrophy was noted (LV: 2060$\pm$154mg, RV: 653$\pm$115mg, n=4, compared to the normals LV: 1291$\pm$127, RV: 433$\pm$32mg, n=3; $P<0.01$ in both cases) although the body mass was diminished (599 $\pm$ 56 versus 700 $\pm$ 35 grams, $P < 0.05$) (8).

Table 1 lists the coefficients of the systolic and diastolic stress-strain relations derived from the corresponding pressure-volume curves (Fig.1). Left ventricles of banded guinea pigs were significantly stiffer compared to controls, as reflected in their steeper diastolic stress-relative strain (Lagrangian) relations (Fig.1D, $P<0.05$). Additionally, their systolic stress-strain (both absolute and relative, as in Figure 1C and 1D respectively) relations were steeper ($P<0.05$), indicating increased active wall stress for comparable stretch of the myocardial wall. Lastly, midwall chamber circumference at zero stress was augmented during hypertrophy (5.12$\pm$0.2cm, n=4) compared to the normal hearts (4.19$\pm$0.04cm, n=3, $P<0.01$). These differences support the characterization of the hypertrophied hearts as being in late-stage congestive heart failure. The steeper systolic stress-strain relations has several possible interpretations, one of which is a change in the sarcomere length-dependence of myofilament calcium sensitivity (14,15). We next investigated the changes in TnT and TnI that accompany myocardial hypertrophy.

Altered distributions of the troponin T isoforms

Immunoblots of the SDS-PAGE were developed for the analysis of troponin T shifts. Figure 2 shows representative troponin T blots for right ventricular samples from a normal

Table 1: COEFFICIENTS OF STRESS-STRAIN RELATIONS

	Midwall Stress – Circumference		Midwall Stress – ΔCircumf.	
	Diastolic	Systolic	Diastolic	Systolic
NORMAL	2.78±0.52	102±24	9.99±1.52	467±188
		*	*	**
HYPERTROPHY/ FAILURE	4.77±1.36	315±94	20.39±6.71	1688±544

Coefficients of systolic and diastolic stress-strain relations relating left ventricular midwall stress to midwall circumference (see Methods and Figure 1C, mmHg/cm) and also to percent change in midwall circumference (ΔCircumf., Figure 1D, mmHg/fractional increase in circumference). Systolic coefficients are the slopes of the linear regressions for the end systolic stress-strain relations. Diastolic stiffness coefficients of the diastolic stress-strain relations (K) were determined by exponential fit of the type: Stress $\equiv e^{K(\text{Strain})} - 1$. Data are for 3 normal hearts and for 4 hypertrophied failing hearts. *$P<0.05$, **$P<0.01$.

guinea pig heart (N1), a hypertrophied failing heart (F1), and another hypertrophied failing heart in which pericardial tamponade was noted (F2). This heart (F2) was exceptional in that prior to vena cava occlusions it had the greatest baseline peak systolic stress (137 mmHg; all others ranged from 42-91 mmHg). In both the normal (N1) and the failing (F1) hearts, four bands (TnT1, TnT2, TnT3, TnT4) are seen. The two higher molecular weight bands (TnT1 & TnT2) had smaller intensity than did the lower two (TnT3 & TnT4) bands. For the normal hearts (n=3), the mean value of the combined intensities TnT3+TnT4 accounted for 61.2±2.2 percent of the total intensity of all four bands (TnT1+TnT2+TnT3+TnT4). In the failing hearts (n=4), the two higher molecular weight bands lost part of their intensities and the combined TnT3+TnT4 intensities accounted for 73.2±14.2 percent of the total.

Interestingly, in the heart with tamponade (F2), the TnT1 and TnT2 bands were relatively undetectable, and TnT3 and TnT4 bands accounted for most (93.7 percent) of the cumulative intensity. Similar observations were made on the left ventricle, in which the percent TnT3+TnT4 was 65.7 in the normal (N1) and increased to 81.1± 6.3% in the hypertrophied failing hearts (n=4). 2-way ANOVA established a significant effect of aortic banding on percent TnT3+TnT4 in these left and right ventricular specimens ($P<0.05$).

Changes in troponin I

We also developed immunoblots using a monoclonal TnI antibody, to describe the possible alterations in TnI in heart failure. In the Western blot (not shown), only one major band

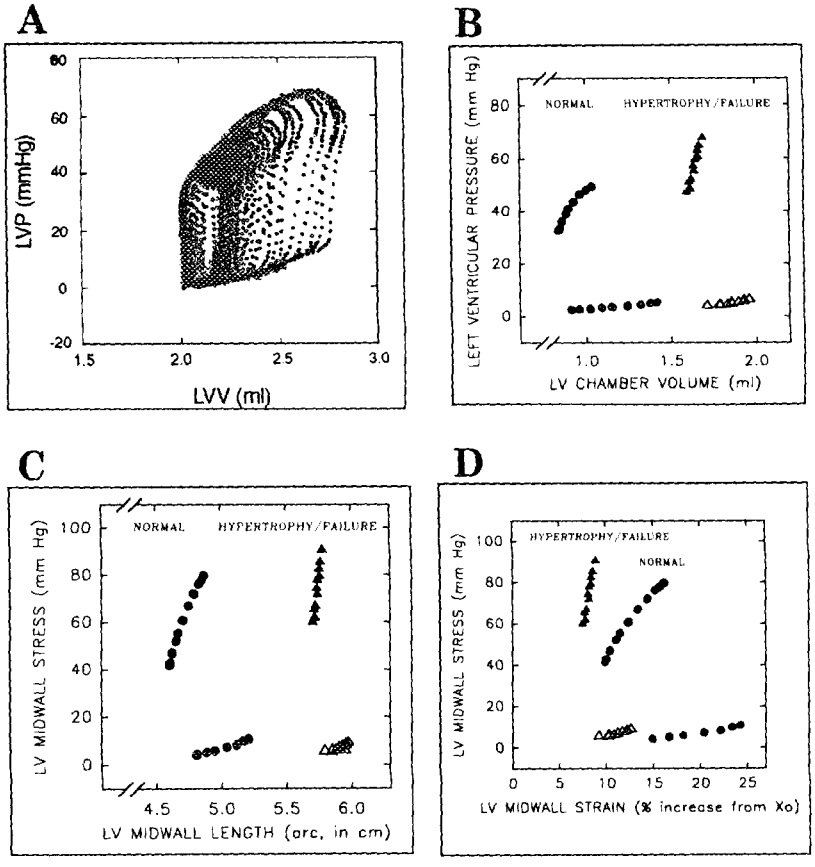
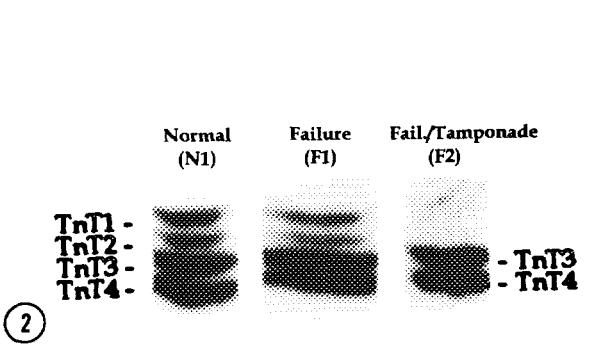


Figure 1. Typical stress-strain analysis in normal (circles) and hypertrophied failing (triangles) guinea pig hearts. A: Left ventricular pressure-volume (P-V) loops in a normal heart *in situ* during a series of vana cava occlusions. B-D: Systolic and diastolic P-V (panel B), and stress-strain (panels C & D) relations (see text).

was resolved for this protein in both normal and failing hearts. This band was quantified on the silver-stained gels (Fig.3). The intensity in TnI band was normalized to myosin light chain 1 in each lane. These estimates (TnI/LC1) in Fig.3 were 42.4% in the normal (N1), 49.5% in failing (F1) and 47.5% in the heart with tamponade (F2). The mean values for all LV and RV specimens were 44.0±2.5% in the normal hearts and 51.9±4.0% in the hypertrophied failing hearts. 2-way ANOVA showed no significant difference between LV and RV specimens, but a significant effect of aortic banding on these values ($P<0.05$). Evidently the fraction of the dominant TnI isoform is increased in the failing heart from the guinea pig, which suggests future investigations for elucidating the underlying mechanisms for this novel observation.

DISCUSSION

These findings provide the first direct evidence in a pressure-overload model of myocardial hypertrophy and failure of altered troponin T and troponin I expressions. Combined



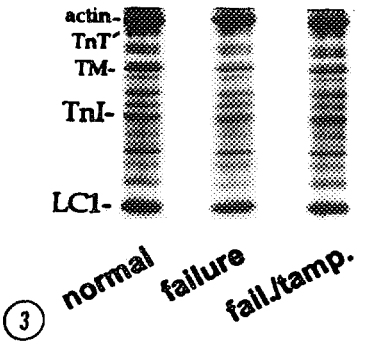


Figure 2. A typical Western blot of troponin T variants in right ventricular specimens from a normal heart (N1) and from two hypertrophied failing hearts (F1 & F2), the latter one of which had pericardial tamponade.

Figure 3. Typical silver-stained gels for specimens of normal, failing and tamponade hearts. Proteins identified are cardiac actin, troponin T, tropomyosin, troponin I, and myosin light chain 1. The TnI band was identified from a parallel Western blot.

with earlier observations in heart failure due to genetic abnormalities or other unknown etiologies (16), our findings suggest that troponin T isoform shifts may generally accompany the development of severe hypertrophy and/or heart failure regardless of the specific etiology.

As many as sixty-four alternately spliced TnT isoforms have been envisaged in skeletal muscle on the basis of highly differentiated intron-exon arrangements of the gene, and several mRNA species coding for these diverse TnT isoforms have indeed been isolated and sequenced (17). Similarly, 12 diverse mRNA species spliced from a unique gene are recognized for rat as well as rabbit cardiac muscle TnT (18,19), and four corresponding protein isoforms from the rabbit have been identified (19). Analogous diversity is also deduced by sequences translated from cDNAs of sheep, cow and chicken hearts.

The possibility that alterations in the troponin-tropomyosin complex affect the Ca-responsiveness of the myofibrils has been raised for congenitally myopathic hamsters (20). Variations in both the myocardial contractile function and the TnT isoform diversity have also been correlated indirectly in a number of independent investigations of the organism during normal development as well as in heart disease. For instance, the relative amount of the common isoform of the newborn rabbit heart missing the most proximal N-terminal exon (so-called cTnT2) has been positively correlated with Ca-sensitivity in myofilaments (21,22). Similar correlations between the variations in TnT isoforms and the contractile sensitivities are also observed in skeletal muscles (23,24). Accordingly, we suggest that the dominant TnT shifts in heart failure in the present study are critical in maintaining contractility in the pressure overloaded guinea pig heart. Interestingly, since corresponding TnT alteration are also observed in the failing hearts in human idiopathic cardiomyopathy (16), the TnT-mediated compensatory

role is very likely a specific response to heart failure more generally. Further investigations of this will be important in future explorations of the molecular basis of these phenomena. Combined molecular physiological studies on isolated tissue specimens with genetically engineered TnT and TnI isoforms, analogous to those made with the TnC subunit (11,25), will be worthwhile for establishing direct functional correlations of the critical exons and should yield specific mechanistic insights.

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