Basic Res Cardiol 92: 147 – 158 (1997)

ORIGINAL CONTRIBUTION

K. Todaka
T. Jiang
J. T. Chapman
A. Gu
S.-M. Zhu
E. Herzog
J. S. Hochman
S. F. Steinberg
D. Burkhoff

Functional consequences of acute collagen degradation studied in crystalloid perfused rat hearts

Received: 6 September 1996
Returned for revision: 8 October 1996
1. Revision received: 17 December 1996
Returned for revision: 7 January 1997
2. Revision received: 20 January 1997
Accepted: 13 February 1997

K. Todaka · J. T. Chapman · A. Gu
S.-M. Zhu · S. F. Steinberg
Dr. D. Burkhoff (✉)
Division of Circulatory Physiology
Milstein Hospital Building Room 5-435
177 Fort Washington Avenue
New York, New York 10032, USA

T. Jiang · S. F. Steinberg
Department of Pharmacology
College of Physicians & Surgeons
of Columbia University
630 West 168th Street
New York, New York 10032, USA

E. Herzog · J. S. Hochman
Division of Cardiology
St. Luke's/Roosevelt Hospital Center
1000 Tenth Avenue
New York, New York 10019, USA

Abstract *Objectives:* The impact of acute collagen disruption by the disulfide donor, 5,5′-dithio-2-nitrobenzoic acid (DTNB) on ventricular properties was tested in rat hearts. *Methods:* Collagen was degraded acutely in 13 isolated, isovolumically contracting rat hearts by perfusion with 1 mM DTNB added to Krebs-Henseleit solution for 1 hour followed by 2-hour perfusion with normal solution. Another 13 hearts were perfused with normal solution for 3 hours (Control). *Results:* Collagen content was 3.5 ± 0.5 % of ventricular dry weight in control group compared with 2.1 ± 0.4 % in DTNB group (decrease by 40 %, $p < 0.01$). Scanning electron micrographs revealed loss of the delicate collagen network surrounding muscle fibers in DTNB treated hearts. Developed pressure at a fixed volume decreased to 86 ± 17 % of the baseline value after 3-hour perfusion in the control group, whereas in DTNB treated hearts developed pressure fell to 68 ± 13 % ($p < 0.01$). End-diastolic pressure was set at 5 mmHg at the beginning of the experiment and rose to 15 ± 8 mmHg in control and 30 ± 13 mmHg ($p < 0.01$) in the treated hearts. Concomitantly, wet-to-dry weight ratio increased from 5.63 ± 0.26 in control to 6.07 ± 0.11 ($p < 0.05$) in the DTNB treated hearts. A separate set of experiments on isolated myocytes excluded the possibility of a direct effect of DTNB on myocyte contractile function. *Conclusions:* These data suggested that with 40 % collagen disruption by DTNB there is a significant increase in tissue edema that results in a decrease in chamber capacitance; in addition, there is a significant decrease in systolic performance which reflects the combined effect of edema and loss of collagen.

Key words Rat – collagen – developed pressure – pressure-volume relationships – extracellular matrix

Introduction

The collagen network of the heart forms an intricate structural weave that interconnects muscle cells, muscle bundles and blood vessels (4, 20, 27). It is believed that collagen strands bear force during systole and limit myocyte excursion during diastole thus implying an important role of collagen in ventricular mechanical pump properties (2, 6, 11, 27, 28).

Collagen degradation has been proposed to contribute to systolic contractile dysfunction in several settings such as in ischemia (26), stunned myocardium (8, 10, 32, 36), and other forms of cardiomyopathy including pacing-induced heart failure (14, 23, 27, 29, 30, 35). However,

Basic Research in Cardiology, Vol. 92, No. 3 (1997)

while there is an association between states of collagen disruption and states of contractile dysfunction, establishment of a causal relationship has not been made. There is no previous study that directly tests the impact of acute collagen disruption on systolic performance and this link remains theoretical.

This may in part be due to the fact that there are relatively few experimental methods available for dissolving the collagen matrix, particularly in a controlled manner such that the degree of degradation mimics what is seen in some clinical settings (8, 27, 31). One previously used method of collagen degradation is to perfuse the heart with a disulfide donor such as 5,5'-dithio-2-nitrobenzoic acid (DTNB) which has been shown to activate endogenous collagenases *in vitro* (7, 24). However, the impact of DTNB perfusion on ventricular performance has not been studied.

Therefore, the purpose of this study was to test the effects of perfusing isolated rat hearts with DTNB on both collagen content and ventricular performance. Biochemical analysis and electron microscopy were used to evaluate the degree of collagen disruption. Ventricular performance was assessed by systolic and diastolic pressures at fixed volumes as well as by measuring pressure-volume relations. The results reveal that the assessment of the pure effects of acute collagen disruption on ventricular performance is complicated by the fact that there is concomitant edema formation which we hypothesize is a direct and unavoidable consequence of acute collagen disruption. Nevertheless, collagen disruption did appear to contribute to systolic contractile dysfunction, though the degree of dysfunction was rather mild considering the significant degree of collagen disruption achieved.

Methods

Isolated rat heart preparation

Thirty-eight Sprague-Dawley rats of either sex weighing 350 – 450 g were heparinized (500 u/kg) and then deeply anesthetized (ketamine 100 mg/kg, xylazine 12.5 mg/kg). After rapid excision of the heart, the aorta was cannulated for retrograde perfusion with a flange-tipped piece of polyethylene tubing over a 16-gauged needle. To create isovolumic contractions, a polyvinyl chloride balloon attached to polyethylene tubing was inserted into the left ventricle (LV) through the mitral valve and held in place by a suture tied around the left atrium. The other end of the tubing was connected to a Statham pressure transducer (model P23XL) for continuous measurement of left ventricular pressure and, via a stop-cock, also connected to a microsyringe for adjustment of left ventricular volume. A second transducer was used to measure coronary perfusion pressure. Pacing was performed with a Grass stimulator (S44) via electrodes attached to the right ventricular outflow tract at a rate above the native sinus rate (180 – 200 /min); pacing rate was held constant throughout the study.

Hearts were perfused with a Krebs-Henseleit (KH) solution that was composed of (in mM): NaCl, 118; $NaHCO_3$, 27.2; KCl, 4.8; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 1.2; $CaCl_2$, 1.5; and glucose, 11.1. Lidocaine (5 mg/l) was also added to suppress ventricular ectopy. This mixture was divided into two portions; DTNB (1 mM) was added to one of these portions. Solutions were stored and warmed to 37 °C in separate containers, equilibrated with 95 % O_2/5 % CO_2, and the pH adjusted to 7.40 throughout the experiment. The flow rate of perfusate was regulated by a rotary pump (Masterflex Model 7523). Perfusate was not recirculated.

The hearts were allowed to stabilize for 30 – 45 min while being perfused by normal KH solution. LV volume (LVV) was adjusted to provide an end-diastolic pressure of 5 mmHg at the beginning of the experiment and LVV was kept constant thereafter. The coronary perfusion flow rate (CF) was adjusted so that initial perfusion pressure was 80 mmHg and CF was kept constant thereafter (control: 10.9 ± 2.3, DTNB: 12.3 ± 2.1 ml/min, NS). Constant perfusion flow was chosen to ensure that ischemia did not occur in the face of myocardial edema (which will be discussed below), although regional ischemia and redistribution of flow cannot be completely excluded. To ensure that severe ischemia was not occurring, we performed histological examination of myocardium at the conclusion of the experiments from 5 of the hearts and found no evidence of myocyte damage, i.e., no contraction band necrosis.

After the stabilization period, control hearts (n = 13) were perfused with KH for 3 hours while LV end-systolic, end-diastolic and developed pressures (ESP, EDP and DP = ESP-EDP respectively) and coronary perfusion pressure (CPP) were recorded at 30 minute intervals. Treated hearts (DTNB group, n = 13) were perfused with Krebs-Henseleit solution with 1 mM DTNB for 1 hour, followed by 2-hour perfusion with normal KH. This procedure has been assumed to induce endogenous collagenolytic activity *in vivo* and has been shown to dissolve the collagen network after a requisite 3 hour perfusion protocol in Langendorf perfused rat hearts (5, 6). At the end of the 3 hour perfusion period, hearts were dismounted from the perfusion apparatus and submitted for analysis of collagen content, wet-to-dry weight ratio or scanning electron microscopy, as detailed below. In 5 of the normal hearts and 5 of the DTNB-treated hearts, ventricular systolic and dias-

tolic properties were further evaluated by measuring the end-systolic and end-diastolic pressure-volume relations (ESPVR and EDPVR, respectively) at the beginning and end of the 3-hour perfusion period. This was accomplished by varying the intraventricular balloon volume over a range which increased LV end-diastolic pressure between 0 and 20 mmHg as has been done previously (3). For comparisons between groups, ESPVRs were quantified by the slope and volume-axis intercept (Ees and Vo, respectively). In order to combine data from different hearts, all volumes were normalized to body weight.

All procedures were in accordance with the institutional guidelines (Institutional Animal Care and Use Committee, Columbia University).

Effects of coronary dilation on ventricular function and collagen content

As will be detailed in Results, DTNB caused significant coronary artery vasodilation, a factor which further complicates the use of this substance to study the functional consequences of collagen degradation. To determine the impact of this factor on the results, we examined the effects of pure coronary dilation on ventricular function and collagen content. The same perfusion protocol described above was used in this series of studies with the exception that adenosine (0.16 ± 0.08 mg/l), instead of DTNB, was added to the perfusate in 6 hearts so that it created comparable coronary dilation as was observed with DTNB and the results were compared to those from 6 additional control hearts. The time course of DP and EDP, collagen content and wet-to-dry weight ratio were measured as in the other groups studied.

Scanning electron microscopy

In order to directly visualize the collagen network, scanning electron microscopy (SEM) was performed. One cubic millimeter pieces of left ventricle from 10 hearts (5 control and 5 DTNB) were fixed overnight with 2 % glutaraldehyde in phosphate buffer and post-fixed in 1 % OsO_4. The tissue was dehydrated in graded concentrations of alcohol, critical point-dried with CO_2, mounted on stubs and then coated with gold. The specimens were photographed with an Amray 1200B scanning electron microscope at a variety of magnifications by an observer who was blinded as to the treatment. Three photographs of each sample (different magnifications of the same site) were examined by two observers that were blinded as to whether samples were taken from control or DTNB-treated hearts. Collagen content by SEM was graded subjectively by the observers as either normal (score = 2), mild-to-moderately decreased (score = 1) or markedly decreased-to-absent (score = 0).

Collagen content by hydroxyproline assay

Since SEM evaluation of the collagen network is qualitative and subject to sampling error, the degree of collagen disruption was quantified using a hydroxyproline assay as described by Stegemann (25, 34) with slight modifications. The left and right ventricles of 8 normal and 7 DTNB-treated hearts were homogenized, hydrolyzed and incubated with Chloramine T. The absorbance of the resulting solution at the wavelength of hydroxyproline chromogen (540 nm) was measured. In order to generate a standardization curve for the measurements, collagen samples of known concentrations were prepared from purified collagen of bovine Achilles tendon and absorbance was measured in the same manner as for the heart samples. Collagen content per dry heart weight was derived in each heart from this standard curve.

Wet-to-dry weight

Wet-to-dry weight ratios (5 control and 5 DTNB-treated hearts) were measured by weighing samples before and after desiccation at 90 °C for 48 hours in a vacuum oven.

Assessment of direct effects of DTNB on myocyte contractile properties

The overall strategy of the present study is to assess mechanical properties of the left ventricle following disruption of the collagen matrix by DTNB treatment. However, if DTNB exerts a direct effect on the mechanical performance of myocytes then it would be impossible to separate the effects of collagen disruption from those of DTNB. In order to test whether DTNB exerts any direct effects on indices of myocardial contractile performances (diastolic cell length, systolic cell length and percent shortening), studies were performed in isolated rat myocytes. Adult rat ventricular myocytes were disaggregated according to methods published previously (16). Cells retrieved from this disaggregation procedure were divided in half and stored in separate test tubes that had or had not been treated with DTNB to permit comparisons of contractile properties of cells isolated from the same heart. The DTNB treatment regimen was designed to mimic the protocol used in the intact heart. Cells were exposed to 1 mM DTNB for 1 hour, washed two times

Basic Research in Cardiology, Vol. 92, No. 3 (1997)

with DTNB-free buffer, and then maintained in DTNB-free buffer for an additional 2 hours. Measurements of resting cell length and cell motion in control and DTNB-treated cells were performed at 30 minute intervals throughout the three hour protocol according to methods published previously (16). In brief, myocytes were taken from the test tube at the designated times, placed in a superfusion chamber, superfused with Tyrode's solution (equilibrated with 95 % O_2 – 5 % CO_2) at a rate of 1 ml/min and paced at 1 Hz by electrical field stimulation using platinum wires. Images of individual cells were projected to a video optical system (Crescent Electronics). The orientation of the image was adjusted so that the position of both ends of the long axis of the cell could be tracked along raster line segments of the image during electrically stimulated contractions. The analogue voltage output from the motion detector was calibrated to convert to microns of motion; motion signals were obtained at a rate of 60 Hz.

Statistics

Data are presented as mean ± SD. Unpaired t-test was used to assess differences in means between control and treated groups. Mann-Whitney rank sum test was used to compare SEM scores for collagen degradation. In order to assess statistical significance of changes in developed pressure and end-diastolic pressure over time, repeated-measures analysis of variance (ANOVA) with Tukey's post hoc test was applied to data from control and DTNB groups separately. An analysis of covariance (ANCOVA) was used to compare ESPVRs. A value of $p < 0.05$ was considered significant.

Results

The effects of DTNB on isolated myocytes

Figure 1 shows the contractile properties of isolated myocytes during and following treatment with control buffer (n = 15, open circles) or buffer containing DTNB (n = 15, filled circles). The upper panel shows the average (± SD) percent cell shortening [(diastolic length-systolic length)/diastolic length × 100], the lower panel shows diastolic cell length. A small increase in percent shortening, indicative of enhanced contractility, was observed in myocytes exposed to DTNB for 45 minutes ($p < 0.01$). This disappeared upon removal of DTNB (after the first hour). Resting cell length did not differ between groups at any time point including during the DTNB exposure period.

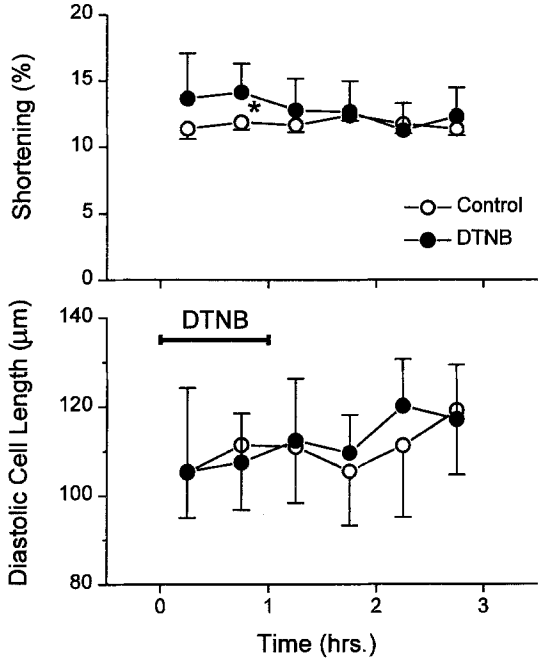

Fig. 1 Percent cell shortening (upper panel), end-diastolic cell length (lower panel) as a function of time in control myocytes (open circles, each observation represents measurements made from 15 cells) and DTNB treated myocytes (filled circles, also from 15 cells at each time point). Treated cells were exposed to DTNB during the first hour (indicated by horizontal bars), followed by normal buffer thereafter. At 45 minutes, there was a significant increase in percent shortening in DTNB group compared to control, which disappeared with removal of DTNB. No other differences between groups were detected. *$p < 0.01$ vs. control by unpaired *t*-test.

These results suggest that DTNB exerts a small positive inotropic effect, but that this effect reverses rapidly when DTNB is removed. Because neither a negative inotropic effect nor a long lasting effect of DTNB was detected, we concluded that this agent is suitable for the purpose of disrupting collagen without interfering directly with myocyte function.

DTNB disrupts the collagen matrix in isolated perfused hearts

Electron micrographs from a representative control heart (panels A, B) and a representative DTNB-treated heart (panels C, D) are shown in Figure 2. As reported previously, there was an extensive delicate collagen network connecting adjacent cardiac muscle fibers and muscle bundles in the control heart, which can be appreciated even at the low magnification (panel A, 200×). At higher magnifications (panel B, 5000×) collagen strands can be

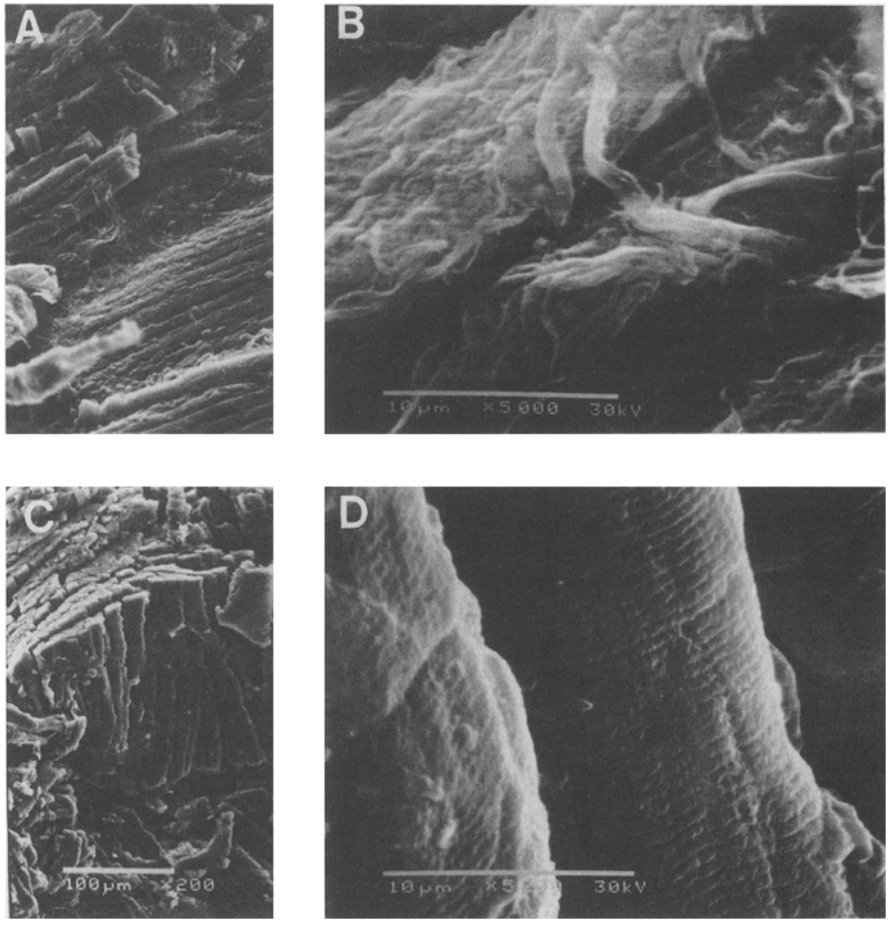

Fig. 2 Scanning electron micrographs of control (panels A, B) and DTNB treated (C, D) myocardium. Original magnifications are: A, C 200×; B 5000×; D 5200×. The delicate collagen network is visualized in control hearts and at higher power is seen surrounding individual muscle cells. In DTNB treated hearts, these features are absent and bare muscle cells are seen. These are representative of 5 normal and 5 DTNB treated hearts.

seen surrounding individual muscle fibers. These features are conspicuously absent from the DTNB-treated heart, where muscle cells can be seen without any obvious collagen strands at low or high power. These findings are representative of 5 normal and 5 DTNB-treated hearts. The mean (± SD) collagen scores given to the control hearts were 1.6 ± 0.5 (observer A) and 1.8 ± 0.5 (observer B), whereas DTNB hearts had scores of 0.6 ± 0.5 (A), 0.4 ± 0.9 (B) ($p < 0.05$ by Mann-Whitney rank sum test, control vs DTNB for both observers).

In order to provide a more objective measure of the degree of collagen disruption, collagen content was quantified by measuring hydroxyproline content in 8 control and 7 DTNB-treated hearts. The results of this analysis, summarized in Figure 3, indicated that collagen accounted for 3.5 ± 0.5 % of the dry weight of normal rat hearts and that this number decreased by 40 % to 2.1 ± 0.4 % in DTNB hearts ($p < 0.01$). Thus, as reported previously (5, 6), there is microscopic and biochemical evidence that 1 hour DTNB administration leads to considerable collagen degradation within 3 hours.

Collagen disruption was associated with decreased LV developed pressure

A comparison between LV developed pressures (DP) from control and DTNB-treated hearts over the 3 hour perfusion period is shown in the upper panel of Figure 4, and a comparison of the EPDs is shown in the lower panel. DP is expressed as a percent of its value at the start of the experiment (time = 0) in order to account for inter-heart variability. Mean (± SD) absolute values (in mmHg) of DP at the start of the protocol were 108 ± 23 in control versus 119 ± 25 in the DTNB group (NS), and values for EDP were 5.1 ± 1.1 in control versus 4.7 ± 1.4 in the DTNB group (NS) indicating that baseline diastolic and systolic properties were comparable in the two groups. DP tended to increase during the period of DTNB exposure suggesting a mild positive inotropic action (similar to that seen in the isolated myocyte studies), though this positive effect did not reach statistical significance ($p = 0.11$ compared to baseline). There was a slight decrease in DP over the perfusion period in the control

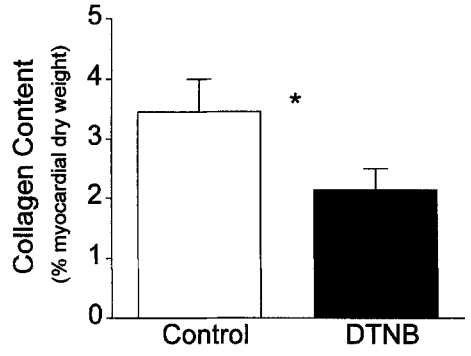

Fig. 3 Collagen content assessed by hydroxyproline assay. There was 40 % less collagen content in DTNB treated hearts than in control. Ventrical bars indicate one SD. n = 8 in control group and n = 7 in DTNB group. * $p < 0.01$ vs. control by unpaired *t*-test.

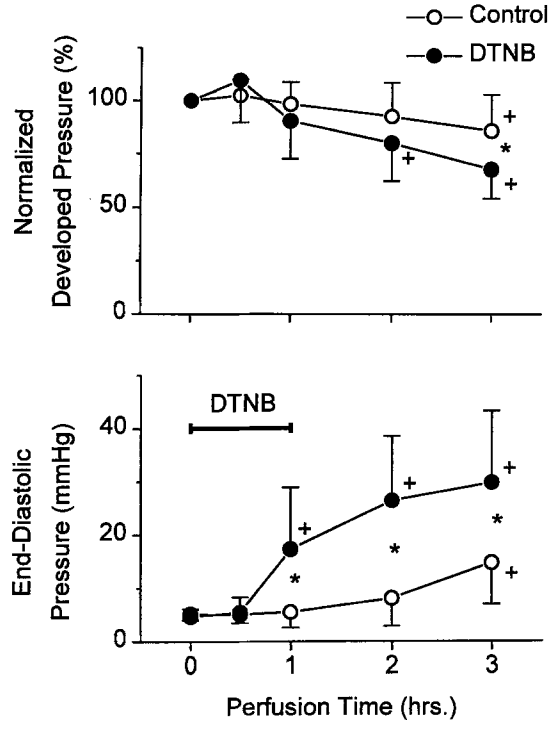

Fig. 4 Isovolumic developed pressure (upper panel) and end-diastolic pressure (lower panel) as a function of time in control hearts (open circles) and DTNB treated (filled circles). In treated hearts, DTNB was infused during the first hour (indicated by horizontal bars), followed by normal Krebs-Henseleit perfusion thereafter. Developed pressure is normalized to its baseline value. At 3 hours of perfusion, there was a significant decrease in developed pressure in DTNB group compared to control. Whereas a marked increase in end-diastolic pressure in treated hearts arose as early as 1 hour. Vertical bars indicate one SD. n = 13 in each group. * $p < 0.01$ DTNB vs. control at each time point by unpaired *t*-test. + $p < 0.01$ compared with the respective baseline by repeated-measures ANOVA and Tukey's procedure.

group ($p < 0.01$ at 3 hours compared to baseline), whereas it decreased to a greater degree in DTNB group ($p < 0.01$ at 2 and 3 hours compared to baseline). After 3 hours of perfusion, the difference between groups was statistically significant: control 86 ± 17 % versus DTNB 68 ± 13 %, $p < 0.01$ (unpaired t-test). Thus DP decreased by an average of 18 % as a consequence of collagen disruption. Absolute values for the final developed pressures were 91 ± 21 mmHg for control group and 78 ± 13 mmHg in the DTNB group ($p = 0.071$ by unpaired t-test). Because of interanimal variability in starting values, we believe that the analysis based upon the normalized developed pressures provides the more relevant information as to the consequences of collagen disruption.

After 3 hours perfusion, EDP increased by an average of 10 mmHg from the starting value in the control group ($p < 0.01$ compared to baseline) wheres it increased by nearly 25 mmHg in the DTNB-treated hearts ($p < 0.01$ compared to baseline). The EDP did not start to increase until between 30 and 60 minutes after commencing DTNB exposure. In contrast, it was not until 2 hours of perfusion that EDP started to rise substantially in control hearts. The differences in EDP between the two groups were statistically significant starting at the one hour time point.

Figure 5 shows how perfusion pressure, measured at constant coronary flow and normalized to the starting value, varied over time. Starting values were 78 ± 4 mmHg

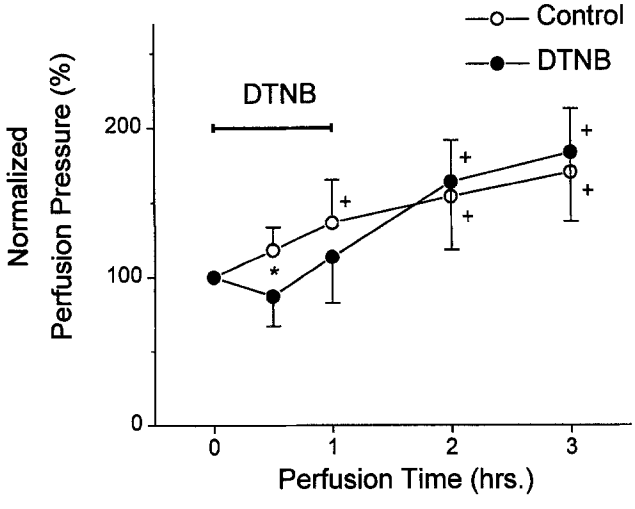

Fig. 5 Normalized coronary perfusion pressure as a function of time in control hearts and DTNB hearts. There was a significant decrease in perfusion pressure during DTNB exposure indicating vasodilatory action; this effect was short-lived. There were no differences between groups after removal of DTNB.

in control and 77 ± 6 mmHg in the DTNB group (NS), indicating compatible starting conditions. At 30 minutes of perfusion, DTNB-treated hearts showed a decreased normalized perfusion pressure which was significantly different from the control hearts, suggesting a mild vasodilating action of DTNB. However, normalized perfusion pressure was the same in the two groups once DTNB was withdrawn after the first hour and remained similar thereafter.

Impact of DTNB on ESPVR and EDPVR

The impact of collagen disruption on the ESPVR and EDPVR are shown for representative control and DTNB-treated hearts in Figure 6. With regard to the ESPVRs, these data show that there was a comparable and small downward shift of the ESPVR in both groups. The results from all hearts are summarized in Table 1, which shows the slope (Ees) and volume axis intercept (Vo) values for each heart at baseline and after 3 hours of perfusion. Statistical analysis (ANCOVA) revealed that there was a significant downward shift of the ESPVR after 3-hour perfusion in both the control and DTNB-treated hearts (manifest as an increase in Vo with no significant change in Ees). The analysis further revealed that there was no difference in ESPVRs between control and DTNB-treated hearts at either time point.

In contrast, the EDPVR shifted upwards only slightly in the control heart but shifted markedly upward in the DTNB-treated heart indicating an overall stiffening of the ventricle and a reduction in diastolic chamber capacitance. These findings were observed consistently in all hearts studied.

Table 1 ESPVRs at the beginning (0 h) and the end of perfusion (3 h)

Rat	Ees	Vo	r^2	Ees	Vo	r^2
Control	0h			3h		
1	231	0.20	0.987	194	0.33	0.994
2	105	–0.62	0.972	142	–0.19	0.998
3	67	–1.22	0.989	96	–0.39	0.985
4	132	–0.24	0.930	184	0.06	0.999
5	170	–0.04	0.992	159	–0.14	0.980
Mean	141	–0.38		155	–0.07*	
SD	63	0.56		39	0.27	
DTNB	0h			3h		
6	68	–1.42	0.955	124	–0.07	0.997
7	117	–0.35	0.995	137	–0.08	0.989
8	202	0.23	0.956	182	0.19	0.982
9	222	0.09	0.991	205	0.03	0.991
10	168	–0.17	0.902	204	0.02	0.996
Mean	156	–0.32		170	0.02*	
SD	63	0.65		38	0.11	

*: $P < 0.01$ significant shift of ESPVR from 0 h by ANCOVA unit for Ees: mmHg·kg/ml, Vo: ml/kg

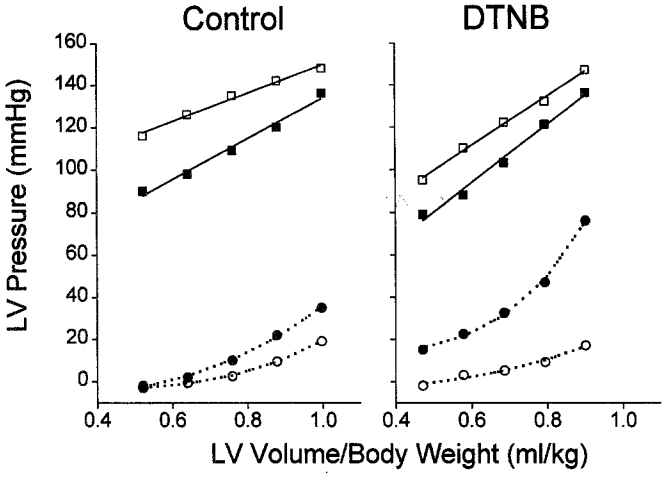

Fig. 6 End-systolic and end-diastolic pressure volume relationships (ESPVRs, EDPVRs) from a representative control heart and a representative DTNB treated heart under control conditions (open symbols) and at the end of the 3 hour perfusion period (filled symbols). Left ventricular (LV) volume was normalized to body weight. Solid lines are ESPVRs by linear regression; dotted lines are EDPVRs fit to a power function. There is a comparable downward shift of the ESPVRs in both groups, but the EDPVR is shifted upward to a much greater degree in the DTNB treated hearts.

DTNB increased myocardial water content

To investigate the potential role of edema formation in explaining the effects on diastolic function, wet-to-dry weight ratios were determined. Results (Fig. 7) showed that the DTNB hearts had greater wet-to-dry weight ratios than in control hearts indicating an increase in water content ($p < 0.01$).

Adenosine did not effect ventricular function, collagen content or myocardial water content

The effects of 1 hour adenosine induced coronary artery dilation on subsequent ventricular performance are summarized in Fig. 8. Adenosine was administered during the first hour to mimic the DTNB protocol. Normalized perfusion pressure at 0.5 and 1 hour decreased significantly compared to control group. However, once the drug was

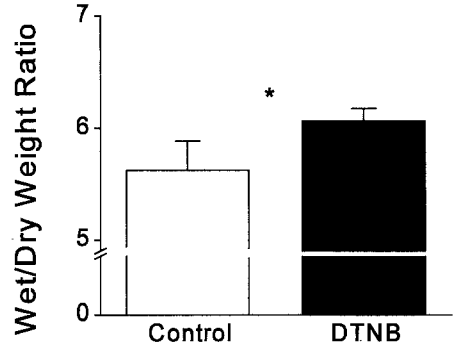

Fig. 7 Wet-to-dry weight ratios of control and DTNB treated hearts. Vertical bars indicate one SD. DTNB treated hearts (with decreased collagen content) had greater water content. * $p < 0.01$ vs. control by unpaired *t*-test. n = 5 in each group.

withdrawn, there was no difference in perfusion pressure between the groups. Importantly, there was no difference in DP or EDP at any subsequent time point despite the significant temporary coronary dilation. Neither collagen content (control: 2.34 ± 0.31 vs. adenosine: 2.42 ±0.26, NS) nor wet-to-dry weight ratio (6.92 ± 0.93 vs. 7.15 ± 0.84, NS) measured at the conclusion of the 3 hour perfusion period were influenced by the adenosine infusion.

Discussion

Consistent with results of previous investigations (5, 6), collagen content in isolated rat hearts was decreased by ~40 % with 1 hour of DTNB perfusion followed by 2 hours of normal KH perfusion. We found that this marked degree of acute collagen disruption resulted in a statistically significant degree (18 %) of contractile dysfunction indexed by developed pressure at a fixed volume in isolated, crystalloid perfused rat hearts. On the other hand, diastolic properties were made stiffer, not more compliant; this was correlated with an increase in myocardial water content following collagen disruption, which may be an unavoidable consequence of collagen disruption. The vasodilatory action of DTNB was excluded as a potential contributing mechanism for these observations in a separate set of experiments in which adenosine was used as a pure coronary vasodilator. Importantly, direct myocardial effects of DTNB were excluded by studying mechanical properties of isolated myocytes during and after DTNB exposure.

The conclusion pertaining to the impact of collagen degradation on systolic strength is based on the use of developed pressure as the contractile index. While the ESPVR has emerged as one of the most commonly used contractile indexes, it has been shown to be insensitive to inotropic state when there are concomitant changes in diastolic properties. This was shown for the case of reperfusion following 90 minutes of ischemia where the EDPVR was elevated, normalized developed pressure at any volume was decreased significantly, but the resulting ESPVR was identical to that measured under control conditions (37). Thus, when there is a decrease in systolic contractile strength, a concomitant elevation of the EDPVR "pushes" the ESPVR back towards its original normal position without truly increasing contractile state. It was

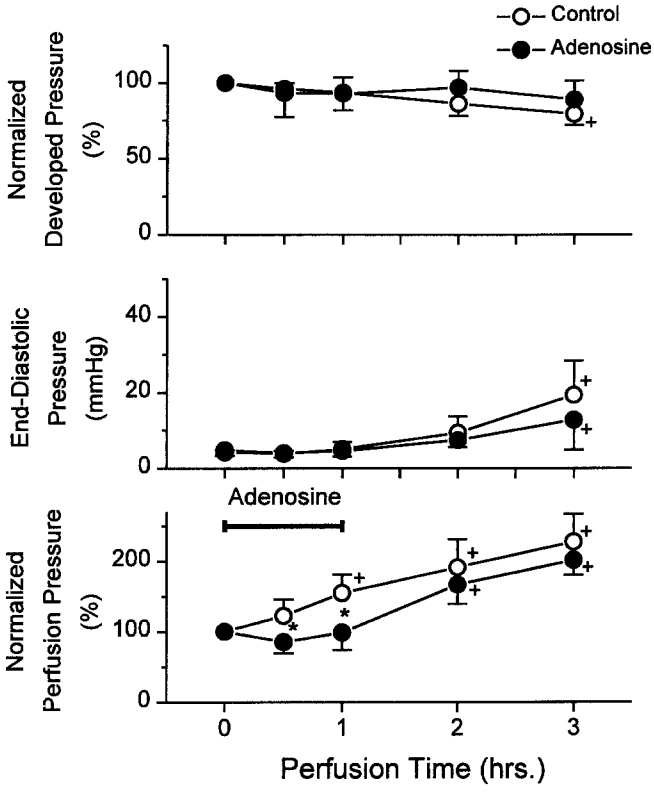

Fig. 8 Isovolumic developed pressure (top panel), end-diastolic pressure (middle panel) and coronary perfusion pressure (bottom panel) as a function of time in control (open circles) and adenosine treated hearts (filled circles). In treated hearts, adenosine was infused during the first hour (indicated by a horizontal bar), followed by normal Krebs-Henseleit perfusion thereafter. Developed pressure and perfusion pressure are normalized to their respective baseline values. There was a significant decrease in perfusion pressure during adenosine exposure indicating vasodilatory action. There was no difference in developed pressure or end-diastolic pressure at any time point in spite of the significant temporary coronary dilation. Vertical bars indicate one SD. n = 6 in each group. * $p < 0.01$ adenosine vs. control at each time point by unpaired *t*-test. + $p < 0.01$ compared with the respective baseline by repeated-measures ANOVA and Tukey's procedure.

proposed that in such cases, developed pressure should be used as an index of systolic function because it is more sensitive to changes in contractile activity than the ESPVR (37). This scenario pertains to the situation observed in the present study where a change in EDPVR was observed following DTNB treatment.

It is also noteworthy that when absolute values of developed pressure were analyzed, the difference between control and DTNB treated hearts was of a smaller value and of borderline significance ($p = 0.07$). Accordingly, it should be noted that if one relied on the analysis of absolute values that a different conclusion could be drawn, i.e., that there is no significant effect of DTNB treatment on systolic function. However, because of interanimal variability in starting values, we believe that the analysis based upon the *normalized* developed pressures provides the more relevant information as to the consequences of collagen disruption.

Because edema formation with the collagen disruption was unavoidable, it is pertinent to question the degree to which the resulting contractile dysfunction was attributable to collagen disruption per se or to the edema itself. It has been shown in previous studies that a large degree of edema causes a depression of developed pressure in Langendorf perfused rat hearts (21) and, conversely, that osmotic compression of myocytes increases tension development (19). However, it is difficult to compare the degree of edema created in the present study with that in previous studies of intact hearts or isolated muscle strips, making an assessment of this question difficult. Our results showing unchanged isolated myocyte function during DTNB superfusion and the adenosine-induced coronary vasodilation experiments suggest that any intracellular edema observed *in vivo* was secondary to the collagen disruption and not to the DTNB itself. Thus, while the effect appears to be due to the collagen disruption, the degree to which the contractile dysfunction relates to a primary or secondary effect of collagen disruption has not been determined.

The importance of collagen in maintaining efficient ventricular contraction and the detrimental consequences of collagen degradation to contractile strength have been alluded to frequently (for example, see refs. (2, 6, 11, 27 28)). The inferential nature of this relationship has become more apparent recently in studies of myocardial stunning. Zhao et al. (36) and Charney et al. (8) imposed repeated, brief periods of coronary artery occlusion in dogs and found decreased collagen content and increased endogenous collagenase activity in the stunned region. They proposed a role of collagen loss in the depressed regional contractility assessed by segmental shortening. Against this hypothesis are the findings of Whittaker et al. (32) who induced different degrees of collagen damage by two stunning protocols and observed comparable decreases in segmental contractile function. This result provided indirect evidence that collagen breakdown does not play an important role in post ischemic contractile dysfunction. Thus, while previous studies examined this question indirectly, the present study showed directly for the first time that a mild degree of contractile dysfunction results from a marked degree of collagen disruption. It is significant that a rather mild degree of systolic dysfunction occurred in response to a large amount of collagen disruption. However, there is no established or even hypothesized relationship between the degree of collagen disruption and the degree of systolic dysfunction. Clearly, further experimental and conceptual work is needed to address this issue.

In contrast to the present results pertaining to the effects of collagen disruption on diastolic properties, MacKenna et al. reported that collagen degradation by bacterial collagenase resulted in a rightward shift of the pressure-volume relationship (17). These studies were performed in arrested rat hearts that were not being actively perfused during the measurement. In addition, the degree of collagen matrix dissolution was significantly less in the previous study than in the present study, and there was no apparent change in water content compared to control hearts. Thus, significant methodologic differences may underlie the differences between that study and the present study.

The impact of collagen disruption on diastolic properties and myocardial edema formation have been investigated in a few previous studies. Kresh measured intramyocardial pressure of rabbit hearts following collagen disruption by DTNB (15). Consistent with our findings, he observed an increase in diastolic tone (assessed by measuring intramyocardial pressure in unloaded crystalloid perfused hearts) and a significant increase in water content; the link between changes in intramyocardial pressures and diastolic ventricular chamber properties was not investigated. In their study of stunned myocardium, Charney et al. (8) found ~13.5 % decrease of collagen content by hydroxyproline assay and a concomitant increase in water content in the affected region. Weber and colleagues noted concomitant collagen degradation and edema formation in the early stages of extracellular matrix remodeling in pressure overload hypertrophy (27) and rapid pacing-induced canine heart failure (14, 23, 30). Other situations in which there is concomitant collagen breakdown and edema formation include myocardial infarction, cardiomyopathy, and myocarditis (9, 13, 31). Though not considered previously, the results of the present study suggest that the collagen disruption may be a primary cause of the edema formation. Armstrong et al. (1) observed an induction of a 92 kD gelatinase which can

degrade type IV collagen, the main constituent of the basement membrane, in the rapid cardiac pacing-induced canine heart failure model. Thus, it can be speculated that edema may result from a loss of vascular integrity following collagen disruption. Alternatively, degraded collagen molecules may increase osmotic pressure which could promote extravasation of water into the interstitium. Thus, the effects of collagen disruption on diastolic function may reflect the balance between two competing phenomena: 1) increased chamber capacitance due to loss of direct mechanical coupling effects with collagen disruption (17) and 2) decreased chamber capacitance due to edema formation.

The duration of collagen disruption may be another factor that determines the impact on diastolic function. For example, Whittaker et al. found loss of collagen struts and a decrease in LV capacitance on the day following a coronary artery ligation and then noted LV dilation in the ensuing days (31). They speculated that this time sequence of change in chamber compliance was due to edema formation and its subsequent resolution, a notion that was supported by histological evidence.

A recent study evaluated the effect of collagen cross-linking inhibition on diastolic function in pigs (12). They showed a decreased diastolic ventricular stiffness by β-aminopropionitril feeding which decreased collagen cross-linking and content. Systolic function indexed by fractional shortening was not changed by the intervention. The discrepancy with the present study may be explained by the above reason, i.e. acute vs. chronic disruption.

As shown in Fig. 5, DTNB-induced coronary vasodilation may have contributed to the higher water content in DTNB group. However, this possibility was excluded by the supplementary adenosine protocol which showed no change in DP, EDP, collagen content or wet-to-dry weight ratio. This is important because it was shown that coronary vasodilation in isolated rat hearts renders the heart more edematous and depresses LV developed pressure (21). Although the degree of coronary dilation achieved in the present study was similar to that achieved in the previous study (~ 20 % decrease in resistance), the other investigators treated the hearts with adenosine for a longer time period at much greater perfusion pressures (as high as 140 mmHg) to specifically induce edema. Thus, that increased edema formation was not observed in the present study with coronary vasodilation reflects methodologic differences between the studies.

Limitations

Edema normally forms in isolated crystalloid perfused hearts with prolonged perfusion due to the low oncotic pressure of crystalloid perfusate. However, the fact that a 3-hour perfusion protocol is necessary to dissolve the collagen matrix by DTNB (5) make this unavoidable. Nevertheless, this limitation does not negate the potential importance of the present findings since, as noted above, there are several settings in which a correlation between collagen disruption and edema formation has been observed.

There has been a report that edema itself can disrupt collagen (18). One may hypothesize that DTNB created interstitial edema first through an unidentified mechanism, and that the edema then caused a disruption of collagen fibers. Although this is a possible scenario, it is unlikely in light of *in vitro* proven collagenase activation by DTNB (24) and the fact that the only other identified effect of DTNB was vasodilation which, when induced even to a greater extent by adenosine, did not cause collagen disruption.

Direct effects of DTNB on myocellular properties have been excluded by studies performed on isolated myocytes. The studies showed, consistent with the studies in the isolated heart, that there was a small direct positive inotropic effect during DTNB exposure, which disappeared when DTNB was withdrawn. Furthermore, DTNB did not induce any long-lasting effects on contractile properties (either end-diastolic length or percent cell shortening). It is still possible that there is a secondary influence of DTNB that may be mdiated by interaction between DTNB and non-myocellular components of the myocardium, but it would seem likely that any such effects are short lived. Finally, breakdown products of collagen degradation may themselves affect myocellular function. These latter two possibilities have not been excluded.

One experimental difficulty in the emerging field of direct studies of collagen disruption on heart function relates to the nature and degree of collagen disruption achieved by different methods. Powerful exogenous collagenases are capable of completely disrupting intercellular connections and reducing the ventricle to a group of disaggregated cells (22). In contrast, endogenous collagenases are much less powerful, though their activation can lead to substantial acute collagen disruption. Therefore, results of functional studies of collagen disruption may vary depending on the type and concentrations of collagenases used. DTNB activates endogenous collagenases *in vitro* (7, 24) and presumably (though never proven) *in vivo* (5). In addition, the degree of collagen disruption seen with DTNB is similar to what has been observed in experimental myocardial infarction (26), though somewhat greater than observed in myocardial stunning (8). Thus, the results of functional studies using DTNB or other agents that activate endogenous collagenases such

as oxidized glutathione (5, 24) may simulate collagen degradation in a pathophysiologically relevant manner and degree. It should be recognized that different results could be obtained if more specific collagenases are used to disrupt specific components of the collagen matrix.

Also, the literature identifies different kinds of collagen fibers in the myocardium: struts, perimysial fibers, epimysial fibers, etc. The present study did not examine the differential effects of DTNB perfusion on these different components. Preliminary studies in our laboratory with silver staining have revealed, however, clumping of large collagen fibers suggesting that they lose their usual connection with the myocytes and recoil. This has two implications. First, it implies that disruption occurs in both the smaller and larger collagen fibers. Second it suggests that the degree of functional impairment in collagen matrix may be much larger than suggested by the hydroxyproline assay. It might be possible that light microscopic techniques such as picrosirius red tissue staining followed by examination with polarized light might be useful in assessing the degree of collagen disruption (33).

Conclusions

Perfusion of isolated rat hearts with DTNB caused acute degradation of the collagen matrix by an average of 40 % and led to systolic dysfunction. However, given the rather large degree of collagen disruption, the impact on systolic function was rather mild. Diastolic properties were made stiffer, likely due to edema formation which we hypothesize is a direct and unavoidable consequence of collagen disruption. These findings therefore imply an important role of collagen degradation in contributing to edema formation and also appear to signify that systolic function may be impaired by collagen disruption, at least to a mild degree.

Acknowledgments This work was supported in part by grants from NHLBI (1R29HL51885-01 and HL49537). K. Todaka was supported by a Post-Doctoral Fellowship from American Heart Association, New York City Affiliate and T. Jiang was supported by an NIH Post-Doctoral Training grant in Pharmacologic Sciences (07271). D. Burkhoff was supported by an Investigatorship award from American Heart Association, New York City Affiliate and a research grant from the Whittaker Foundation.

References

1. Armstrong PW, Moe GW, Howard RJ, Grima EA, Cruz TF (1994) Structural remodelling in heart failure: gelatinase induction. Can J Cardiol 10: 214–220
2. Borg TK, Ranson WF, Moslehy FA, Caulfield JB (1981) Structural basis of ventricular stiffness. Lab Invest 44: 49–54
3. Burkhoff D, Weiss RG, Schulman SP, Kalil-Filho R, Wannenburg T, Gerstenblith G (1991) Influence of metabolic substrate on rat heart function and metabolism at different coronary flows. Am J Physiol 261 (Heart Circ Physiol 30): H741–H750
4. Caulfield JB, Borg TK (1979) The collagen network of the heart. Lab Invest 40: 364–372
5. Caulfield JB, Wolkowicz PE (1988) Inducible collagenolytic activity in isolated perfused rat heart. Am J Pathol 131: 199–205
6. Caulfield JB, Wolkowicz PE (1990) A mechanism for cardiac dilation. Heart Failure 6: 138–150
7. Cawston TE, Murphy G (1981) Mammalian collagenases. Methods in Enzymology 80: 711–722
8. Charney RH, Takahashi S, Zhao M, Sonnenblick E, Eng C (1992) Collagen loss in stunned myocardium. Circulation 85: 1483–1490
9. Dec GW, Palacios IF, Fallon JT, Aretz T, Mills J, Lee DC, Johnson RA (1985) Active myocarditis in the spectrum of acute dilated cardiomyopathies – clinical features, histologic correlates, and clinical outcome. N Engl J Med 312 (14): 885–890
10. Eng C, Zhao M, Factor SM, Sonnenblick EH (1993) Post-ischemic cardiac dilatation and remodeling: reperfusion injury of the interstitium. Europ Heart J 14 (Supplement A): 27–32
11. Factor SM, Robinson TF (1988) Comparative connective tissue structure-function relationships in biologic pumps. Lab Invest 58: 150–156
12. Kato S, Spinale FG, Tanaka R, Johnson W, Cooper GCI, Zile MR (1995) Inhibition of collagen cross-linking: effects on fibrillar collagen and ventricular diastolic function. Am J Physiol 269 (HCP 38): H863–H868
13. Kodama M, Hanawa H, Zhang S, Saeki M, Koyama S, Hosono H, Miyakita Y, Kotoh K, Inomata T, Izumi T, Shibata A (1993) FK506 therapy of experimental autoimmune myocarditis after onset of the disease. Am Heart J 126: 1385–1392
14. Komamura K,Shannon RP, Ihara T, Shen Y, Mirsky I, Bishop SP, Vatner SF (1993) Exhaustion of Frank-Starling mechanism in conscious dogs with heart failure. Am J Physiol 265 (Heart Circ Physiol 34): H1119–H1131
15. Kresh JY (1993) Intramyocardial mechanical states: vessel-interstitium-muscle inface. In: Interactive Phenomena in the Cardiac Sstem, edited by S. Sideman and R. Beyar. New York: Plenum Press, p 113–123
16. Kuznetsov V, Pak E, Robinson RB, Steinberg SF (1995) β_2-adrenergic receptor actions in neonatal and adult rat ventricular myocytes. Circ Res 76: 40–52
17. MacKenna DA, Omens JH, McCulloch AD, Covell JW (1994) Contribution of collagen matrix to passive left ventricular mechanics in isolated rat hearts. Am J Physiol 266 (Heart Circ Physiol 35): H1007–H1018
18. Matsubara BB, Henegar JR, Janicki JS (1992) Functional and morphological consequences of induced myocardial collagen damage. Circulation 86: I-171 (Abstract)

19. McDonald KS, Moss RL (1995) Osmotic compression of single cardiac myocytes eliminates the reduction in Ca^{2+} sensitivity of tension at short sarcomere length. Circ Res 77: 199–205
20. Robinson TF, Cohen-Gould L, Factor SM (1983) Skeletal framework of mammalian heart muscle: arrangement of inter-and pericellular connective tissue structures. Lab Invest 49: 482–498
21. Rubboli A, Sobotka PA, Euler DE (1994) Effect of acute edema on left ventricular function and coronary vascular rsistance in the isolated rat heart. Am J Physiol 267 (Heart Circ Physiol 36): H1054–H1061
22. Sakai M, Danziger RS, Staddon JM, Lakatta EG, Hansford RG (1989) Decrease with senescence in the norepinephrine-induced phosphorylation of myofilament proteins in isolated rat cardiac myocytes. J Mol Cell Cardiol 21: 1327–1336
23. Spinale FG, Tomita M, Zellner JL, Cook JC, Crawford FA, Zile MR (1991) Collagen remodeling and changes in LV function during development and recovery from supraventricular tachycardia. Am J Physiol 261 (Heart Circ Physiol 30): H308–H318
24. Springman EB, Angleton EL, Birkedal-Hansen H, Van Wart HE (1990) Multiple mode of activation of latent human fibroblast collagenases: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc Natl Acad Sci 87: 364–368
25. Stegemann H (1958) Mikrobestimmung von Hydroxyprolin mit Chloramin-T und p-Dimethylaminobenzaldehyd. Hoppe-seyleis 2 Physiol Chemie 311: 41–45
26. Takahashi S, Barry AC, Factor SM (1990) Collagen degradation in ischaemic rat hearts. Biochem J 265: 233–241
27. Weber KT (1989) Cardiac interstitium in health and disease: The fibrillar collagen network. J Am Coll Cardiol 13: 1637–1652
28. Weber KT, Janicki JS, Shroff SG, Pick R, Chen RM, Bashey RI (1988) Collagen remodeling of the pressure overloaded, hypertrophied nonhuman primate myocardium. Circ Res 62: 757–765
29. Weber KT, Pick R, Janicki JS, Gadodia G, Lakier JB (1988) Inadequate collagen tethers in dilated cardiomyopathy. Am Heart J 116: 1641–1646
30. Weber KT, Pick R, Silver MA, Moe GW, Janicki JS, Zucker IH, Armstrong PW (1990) Fibrillar collagen and remodeling of dilated canine left ventricle. Circulation 82: 1387–1404
31. Whittaker P, Boughner DR, Kloner RA (1991) Role of collagen in acute myocardial infarct expansion. Circulation 84: 2123–2134
32. Whittaker P, Boughner DR, Kloner RA, Przyklenk K (1991) Stunned myocardium and myocardial collagen damage: differential effects of single and repeated occlusions. Am Heart J 121: 434–441
33. Whittaker P, Kloner RA, Boughner DR, Pickering JG (1994) Quantitative assessment of myocardial collagen with picrosirius red staining and circularly polarized light. Basic Res Cardiol 89: 397–410
34. Woessner JF (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 93: 440–447
35. Yoshikane H, Honda M, Goto Y, Morioka S, Ooshima A, Moriyama K (1992) Collagen in dilated cardiomyopathy – scanning electron microscopic and immunohistochemical observations. Jpn Circ J 56: 899–910
36. Zhao M, Zhang H, Robinson TF, Factor SM, Sonnenblick EH, Eng C (1987) Profound structural alterations of the extracellular collagen matrix in postischemic dysfunctional ("stunned") but viable myocardium. J Am Coll Cardiol 10: 1322–1334
37. Zile MR, Izzi G, Gaasch WH (1991) Left ventricular diastolic dysfunction limits use of maximum systolic elastance as an index of contractile function. Circulation 83: 674–680