Collagen Loss in the Stunned Myocardium

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Background. This study was performed to biochemically assess and quantify the previously observed ultrastructural alterations in the collagen matrix of stunned myocardium.

Methods and Results. The stunned myocardium was produced in 13 mongrel dogs by a series of 12 coronary artery occlusions of 5 minutes followed by 10-minute reperfusion periods, with a final reperfusion period of 90 minutes. Regional systolic function in the stunned myocardium was 17% of control. Relative end-diastolic length in the stunned region increased up to 8%. There was a nonuniform transmural loss of collagen. Hydroxyproline in the stunned endocardium was not different from control. The stunned midwall and epicardium demonstrated 12.5% (p<0.05) and 14.6% (p<0.005) decreases, respectively. All transmural layers in the stunned myocardium had significant increases in collagenase activity before procollagenase activation, averaging a 73.6% increase (p<0.025). Complete activation of all procollagenase forms with aminophenylmercuric acetate revealed no differences in fully activated collagenase between the stunned and normal regions. The lysosomal enzymes, elastase and cathepsin G, were not different between stunned and normal zone tissue. These results would tend to exclude exogenous sources of protease in the stunned myocardium at the 90-minute final reperfusion time frame. Collagen fibers were isolated from the stunned and normal zone tissue and underwent dansyl chloride reaction. Stunned collagen fibers had 9% greater dansyl labeling, suggesting greater numbers of exposed N-terminal amino acid residues on the fiber and compatible with greater enzymatic cleavage activity on the stunned collagen matrix. Tissue water content was consistently greater in the stunned region compared to the normal: a uniform transmural increase of approximately 1.7%.

Conclusions. The stunned myocardium is characterized by both systolic dysfunction and diastolic expansion or dilatation. Endogenous procollagenase is activated by the ischemic process leading to degradation of the extracellular matrix. The underlying mechanisms may be relevant in ischemic enlargement of the heart and cardiomyopathy. (Circulation 1992;85:1483–1490)

KEY WORDS • collagen • collagenase • myocardial ischemia

Brief coronary artery occlusion, insufficient to cause cell necrosis, nevertheless results in a dysfunctional myocardium. Mechanical and certain biochemical abnormalities may persist for up to 1 week, and the delayed recovery of cardiac contractile function has been termed "stunned myocardium." We have identified profound ultrastructural changes in the extracellular collagen matrix of the stunned myocardium. Since the extracellular matrix may serve as a coupling mechanism for transmission of active and passive forces within the heart, degradation of this matrix during ischemia could have important impact on both systolic and diastolic function. The purpose of this study was to quantify the amount of collagen lost and to assess whether there are transmural patterns of degradation in the stunned myocardium. Collagen content was evaluated by measuring hydroxyproline, an amino acid unique to collagen representing approximately 10% of collagen's amino acid content. Tissue collagenase, elastase, and cathepsin G activities were determined in both the stunned and the normal myocardium. Finally, indirect evidence for in vivo enzymatic cleavage of the collagen matrix was assessed by reacting isolated tissue collagen fibers with dansyl chloride.

Methods

Animal Preparation

Fifteen mongrel dogs of either sex weighing 10–29.5 kg were used in this study. The dogs were anesthetized with intravenous sodium pentobarbital (30 mg/kg body wt). Additional pentobarbital was administered as required to maintain appropriate anesthesia. The dogs were intubated and placed on an intermittent positive-pressure respirator with oxygen supplementation. A femoral cutdown was performed, and fluid-filled catheters were introduced into the femoral artery and vein. The arterial catheter was passed retrograde into the thoracic aorta to measure systemic pressure using a Statham P23Db transducer. A thoracotomy was performed in the fifth left intercostal space. The heart was exposed in a pericardial cradle.
The coronary anatomy was examined, and either the left anterior descending (LAD) or left circumflex (LCx) coronary artery was dissected free proximal to any major branches. A 7F catheter-tip pressure transducer (Millar, Houston, Tex.) was inserted into the left ventricle via a stab wound in the apex to measure left ventricular pressure, left ventricular dP/dt, and high-gain left ventricular diastolic pressure.

Regional myocardial function was measured by sonomicroscopy. Pairs of ultrasonic crystals (1.5 mm in diameter) connected to an ultrasonic dimension system (model 401, Scheussler and Associates) were implanted in the subendocardium, at midventricle, of both the LAD and LCx coronary artery perfusion regions. They were positioned approximately 15 mm apart and oriented in a circumferential direction. All physiological data were recorded on a Beckman type SII multichannel recorder.

**Stunning Protocol**

After stabilization of hemodynamics, control data were recorded. The designated coronary artery was occluded for 5 minutes with a silk ligature snare; this was followed by a 10-minute reperfusion period. Data recordings (100 mm/sec) were taken at end expiration for each reperfusion period just before the next coronary occlusion. This occlusion–reperfusion process was repeated 12 times. Upon completion of the 12th reperfusion, an additional 90-minute reperfusion period was allowed before termination of the experiment. Arterial blood gases were monitored for pH, PaO2, and PaCO2 on a blood gas system (model BM53MK2, Radiometer Copenhagen). Respiratory tidal volume and rate were adjusted, and sodium bicarbonate was administered as needed to maintain physiological parameters.

**Tissue Sampling and Hydroxyproline Measurement**

Upon completion of the 90-minute reperfusion period, a lethal dose of KCl was administered intravenously. The heart was removed, and the normal and stunned regions were topically demarcated according to the vessel distribution. The ventricle was sliced from apex to base in 6–10-mm-thick rings. Two transmural tissue samples each were taken from the center of the normally perfused region and from the stunned region. Tissue from the septum was not sampled. Areas where the ultrasonic crystals had been implanted were avoided. The transmural tissue samples were trimmed of the innermost and outermost 1 mm of tissue and divided into three sections: endocardial, midwall, and epicardial layers. Each sample was weighed on a Mettler scale (type H6T) with accuracy to 0.1 mg. Samples approximately 0.5 g wet wt were dried overnight in a vacuum oven at 60°C. The samples were reweighed and placed in Pyrex culture tubes (No. 9826) to which 6N hydrochloric acid was added. The capped tubes were incubated at 116°C for at least 16 hours to ensure total hydrolysis.

A modified version of the hydroxyproline assay described by Berg in which hydroxyproline is converted to pyrrole and measured colorimetrically was employed. Briefly, a 4.0-ml aliquot of a 26.7-fold dilution of the hydrolysate was neutralized with potassium hydroxide and saturated with potassium chloride. One-half milliliter of a 10% alanine solution and 1.0 ml potassium borate buffer were then added, and the samples were mixed and allowed to stand for 30 minutes. The addition of 1.0 ml of a freshly made solution containing 0.2 M chlorine-T dissolved in ethylene glycol monoethylether converted hydroxyproline to pyrrole-2-carboxylic acid by oxidation. This reaction was terminated by addition of 3.0 ml of 3.6 M sodium thiosulfate after 25 minutes. After nonspecific toluene extraction, the samples were placed into a boiling water bath for 30 minutes to convert the pyrrole-2-carboxylic acid to pyrrole. After cooling, the pyrrole was specifically extracted with 5.0 ml toluene. A 2.0-ml sample of the toluene extract was placed in another tube to which 0.8 ml of Ehrlich’s reagent (p-dimethylaminobenzaldehyde) was added, mixed, and allowed to incubate for 30 minutes. The optical density was measured on a spectrophotometer (model 25, Beckman) at A560 nm. A blank and a 10 μg hydroxyproline standard were included in each assay. Tissue hydroxyproline was expressed in micrograms hydroxyproline per milligram dry tissue weight (μg/mg dry wt). Values from the two separate tissue measurements within each layer from each respective normal or stunned region were averaged.

**Tissue Protease Measurements**

In four experiments, the animals underwent the stunning protocol described above. At the end of the final 90-minute reperfusion period, the heart was arrested with a concentrated KCl solution. The heart was then removed along with a segment of the ascending aorta. The aorta was cannulated with a large-bore cannula, and the heart was perfused with 500 ml chilled potassium phosphate buffer (pH 7.3–7.4). This was performed to remove plasma protease inhibitors. Transmural samples from the normal and stunned regions were taken, divided into endocardial, midwall, and epicardial pieces, and frozen on dry ice. They were stored at −70°C until the day of the assay. Production of samples and protease assays were performed as previously described with modifications. Approximately 200 mg of tissue was suspended in 1 ml of 10 mM Tris-HCl buffer containing 0.1% Triton X-100 and 0.02% NaN3, pH 7.5. The tissue was sonicated for 15 seconds at 4°C (Branson Sonicator model W-220 F, Heat Systems-Ultransonic, Inc.) with a control setting of 6. A fiber (containing more than 95% of the collagen in the tissue) adherent to the sonicator microtip was shaved and processed for dansyl chloride reaction as described below. The homogenate was centrifuged at 105,000g for 1 hour at 4°C, and the resulting supernatant was used for protease assays.

Enzyme assays were performed in duplicate with appropriate controls. Tissue collagenase activity was measured with and without activation of pro-collagenase by aminophenylmercuric acetate (APMA) (0.5 mM in 50 mM Tris-HCl buffer, pH 7.5) for 3 hours at 37°C. The reconstituted collagen fibril assay contained [14C]glycine-labeled guinea pig type I collagen as substrate (3,000 cpm/100 μg assay) in buffer containing 2.5 mM disopropylphosphorofluoridate and 0.02% NaN3 at 37°C. Tissue collagenase activity was normalized and expressed in units per hour per 100 mg protein, where 1 unit of collagenase degrades 1 μg of native collagen at 37°C.
Elastolytic activity was measured using a synthetic substrate for elastase, Suc-(Ala)₃-p-nitroanilide. Our previous study demonstrated a direct correlation between synthetic and native substrate for the elastase assay. Fifty microliters each of the substrate (1 mg/ml), buffer (0.1 M Tris-HCl), 10% dimethylsulfoxide (DMSO), and tissue homogenate were reacted for 8 hours at 37°C in the dark, pH 7.5. The reaction was terminated with 0.8 ml of 1.5 M acetic acid. Enzyme activity was expressed in units per hour per 100 mg protein, where 1 unit of elastase is the amount that releases 1 µmol of p-nitroaniline at 37°C. Absorbance for the latter was measured at A410 nm (extinction coefficient, 8,800/M/cm). Cathepsin G activity was measured using a synthetic substrate, Suc-(Ala)₃-Pro-Phε-p-nitroanilide. Fifty microliters each of the substrate (11 mg/ml), 10% DMSO, tissue homogenate, and 100 µl of buffer (0.1 M HEPES/0.5 M NaCl) were reacted for 29 hours at 37°C, pH 7.5. The reaction was terminated with 0.8 ml of 1.5 M acetic acid. Enzyme activity was expressed in units as described for elastase. Tissue protein was determined by measuring amino acid content using the ninhydrin reaction of acid hydrolysates (6N HCl, at 116°C for 24 hours) with leucine serving as a standard and assuming 10 µmol Leu equals 1 mg protein.

Dansylation of Isolated Tissue Collagen Fibers

The collagen fiber from the original sonication (described above) was further sonicated at 4°C for a total of 120 seconds (15-second sonications repeated seven times at 45-second intervals). The final pellet (containing more than 85% of the original collagen with about a 20-fold purification) was washed with distilled water and freeze-dried. Dansylation of the collagen fiber was performed by the method of Gray with the following modifications. The dried collagen fiber (1 mg) was placed into an Eppendorf tube, and 0.8 ml of saturated NaHCO₃ and 0.2 ml of 1% dansyl chloride in acetone were added (pH 8.5). The mixture was reacted in the dark for 24 hours at 25°C. After the reaction, the dansylated collagen fiber was collected by centrifugation. Excess dansyl chloride was removed by extensive washing with water, acetonitrile, and acetone until the wash solution showed no detectable fluorescence. The fiber was then acid-hydrolyzed (6N HCl) in an evacuated sealed tube at 105°C for 18 hours. Before the fluorometric assay, all samples were Millipore-filtered. The fluorescent intensity of the hydrolysate was measured on a fluorescent spectrophotometer (Perkin-Elmer model 650-10S) with an excitation at 340 nm and an emission at 530 nm under ambient temperature. Hydroxyproline content of an aliquot of the fiber hydrolysate was determined by high-performance liquid chromatography as described previously. The intensity of fluorescence was normalized to the hydroxyproline content of the fiber and expressed as E340/mmol Hyp.

Data Analysis

Regional myocardial function was determined by measurement of regional segment length changes in the normal and stunned regions. End-diastolic length (EDL) was measured at the time of initial rise of the first derivative of left ventricular pressure (dP/dt), and the end-systolic length (ESL) was considered to be at peak negative left ventricular dP/dt. Regional myocardial function was expressed as percent systolic shortening (%SS)=100×[EDL−ESL]/EDL, percent control systolic shortening=(100×[%SS]/control %SS), percent control EDL=(100×[EDL−EDLC]/EDL), and percent change in EDL=(100×[EDL−EDLC]/EDL), where EDL represents the EDL at baseline control, and EDLC represents the EDL at a given reperfusion period.

To account for the variability in end-diastolic pressure during the experimental protocol, a ratio of the simultaneous percent control EDL in both regions was calculated. This ratio (expansion index) is considered to be an index of the relative increase in EDL of the stunned myocardium over and above any change in the normal region.

Statistical Analysis

Data are presented as mean±S.D., except in the figures where SEM values are used. A two-way ANOVA with repeated measures was used to assess regional myocardial function data. The grouping factor was coronary artery ligated (LAD and LCx), and the within factor was reperfusion number (baseline plus the 12 reperfusion periods). A two-way ANOVA with repeated measures was used to evaluate hydroxyproline and water content data. The grouping factor was coronary artery ligated, and the two within factors were region (normal versus stunned) and transmural location (endocardium, midwall, and epicardium). A two-way ANOVA with repeated measures was performed on the collagenase, lysosomal enzyme activities, and dansylation data. A post-hoc Newman-Keuls test was performed on cell means of interest for significant overall F values. A value of p<0.05 was considered significant.

Results

Two dogs were excluded from the data analysis; one dog experienced ventricular fibrillation that was refractory to direct current cardioversion, and in another dog, a technical problem was encountered during tissue hydrolysis with loss of hydrolysate volume. The results represent data from 13 dogs, with stunning of the LAD territory in six and stunning of the LCx territory in seven dogs. Two dogs experienced ventricular fibrillation that responded to low-voltage DC cardioversion and are included in the analysis.

Hemodynamics

The hemodynamics are summarized in Table 1. There was a progressive decline of the heart rate from 137±26 to 118±31 beats per minute during the course of the 4.5-hour protocol (p<0.001, overall ANOVA). The aortic pressure demonstrated a small, nonsignificant (p=0.07) rise over the course of the protocol. The left ventricular end-diastolic pressure decreased from 8.4±3.5 to 5.1±2.9 mm Hg (p<0.001, overall ANOVA) after a transient initial rise in the first reperfusion period.

Regional Function

Significant systolic dysfunction was present in the stunned myocardium (Figure 1). The percent segment shortening was 15.3±8.5% at control, decreased to 7.3±8.0% during the first reperfusion period, and decreased further to 2.6±11.5% by the end of the final 90-minute reperfusion period. The latter represents 16.9% of control systolic function. Systolic function in


<table>
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<th>Reperfusion</th>
<th>HR (bpm)</th>
<th>AP (mm Hg)</th>
<th>LVEDP (mm Hg)</th>
<th>Stunned (% SS)</th>
<th>Normal (% SS)</th>
<th>Stunned (% change in EDL)</th>
<th>Normal (% change in EDL)</th>
<th>Relative expansion index (%)</th>
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Values represent mean±SD (n=13). C, control baseline; HR, heart rate (significant decrease in heart rate occurred over the course of the 12 occlusion–reperfusion protocol, p<0.001); bpm, beats per minute; AP, aortic pressure (slight increase in aortic pressure did not achieve statistical significance, p=0.07); LVEDP, left ventricular end-diastolic pressure (significant decrease in LVEDP occurred during the course of the protocol, p<0.001); %SS, percent systolic shortening (there were significant differences in systolic function between the stunned and normal myocardium, p<0.001, with significant loss of systolic function in the stunned myocardium, p<0.001, interaction effect on ANOVA); % change in EDL, changes in end-diastolic length in the stunned myocardium were significantly different from those in the normal zone (p<0.001, ANOVA); Relative expansion index, represents the percent of control EDL in the stunned myocardium relative to the simultaneous normal zone EDL at a given reperfusion period.

the normal zone was preserved and slightly above control throughout the protocol. The percent segment shortening was significantly different between ischemic and normal regions (p<0.01, ANOVA) with a significant interaction effect (p<0.001). There were no significant differences in the amount of systolic dysfunction produced by LAD or LCx occlusions.

Both the normal and stunned regions demonstrated increased EDL (Figure 2 and Table 1). The increase in EDL in the normal region was brief and returned to near baseline by the fourth reperfusion period. In the stunned region, the EDL continued to increase until the sixth reperfusion, where it plateaued. The increase in EDL in the stunned region was consistent and highly significant (p<0.001, overall ANOVA). The EDL values in both regions were below control EDL at the final 90-minute reperfusion period. This was presumably due to a significant decrease in the left ventricular diastolic pressure. To account for variability in preload during the protocol, the relative expansion index was used. At any given reperfusion period, the normal and stunned regions experience the same end-diastolic pressure. Thus, this index is considered to represent increases in stunned EDL over and above any changes in normal zone EDL. Relative to the normal region, there was a 5–8% increase in stunned zone linear dimension that roughly translates into a threefold or 15–25% increase in regional volume (Table 1 and Figure 2).

**Myocardial Edema**

Tissue in the normal region contained 75.5±1.0% H₂O. Tissue in the stunned region contained 76.8±1.5% H₂O. This increase in water content in the ischemic region was highly significant (p<0.005, overall ANOVA). The endocardial, midwall, and epicardial regions of the stunned myocardium all showed greater water content compared with their respective samples from the normal region, with increases of 1.60%, 1.82%, and 1.75%, respectively (p<0.001). There were no transmural differences in edema. Ischemia of the LAD and LCx regions resulted in comparable increases in water content: 1.84% and 1.60%, respectively.

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**Figure 1.** Plot of percent systolic shortening (n=13). Segment shortening in the stunned region was 15.3±2.4% (SEM) at control baseline and 2.6±3.2% at completion of the protocol. Segment shortening in the normal region was 13.5±1.5% at baseline and 13.9±2.1% at completion of the protocol. Reperfusion number indicates measurements performed at the end of each reperfusion period. Function in the stunned region was significantly different from the normal zone (p<0.01, ANOVA) with a significant interaction effect (p<0.001, ANOVA).
Protease Activities

Tissue from the stunned region had significantly greater collagenase activity than the normal region before procollagenase activation by APMA (p = 0.03, overall ANOVA). Collagenase activity in the stunned endocardial, midwall, and epicardial layers was 5.13, 4.88, and 6.80 units/hr/100 mg protein, respectively, compared with 2.52, 2.78, and 4.38 units/hr/100 mg protein in the normal zone endocardial, midwall, and epicardial layers (Figure 3 and Table 2). The stunned myocardium demonstrated 55.4%, 75.7%, and 103% increases in collagenase activity for the epicardial, midwall, and endocardial layers, respectively (p < 0.025 for each layer). Total collagenase activity following procollagenase activation by APMA was not significantly different in the normal or stunned regions, nor between layers. Table 2 summarizes the tissue activity of elastase and cathepsin G in the normal and stunned regions. There were no significant differences between the normal and stunned myocardium for both of these lysosomal enzymes, and no transmural differences were found.

Collagen Fiber Dansylation

Dansyl emission, which assesses exposed N-terminal amino acid residues in the isolated fiber, was significantly increased in the stunned region (1.79±0.12 units/mmol Hyp) compared with the normal region (1.65±0.10 units/mmol Hyp) (p < 0.01). All layers of the stunned myocardium had greater dansyl chloride labeling. Dansyl emissions in the stunned region compared with the normal region were 1.77±0.16 versus 1.68±0.10 units/mmol Hyp (endocardium), 1.79±0.08 versus 1.60±0.09 units/mmol Hyp (midwall), and 1.82±0.15 versus 1.67±0.11 units/mmol Hyp (epicardium). The stunned-to-normal-zone ratios of dansyl labeling for the endocardial, midwall, and epicardial regions were 1.06±0.10, 1.12±0.09, and 1.09±0.06, respectively.

Discussion

The major findings of this study are that 1) a significant 5–8% relative increase in EDL (expansion) occurred in the stunned myocardium; 2) a nonuniform transmural loss of collagen occurred in the stunned tissue associated with a 73.6% increase in collagenase activity; 3) APMA-activated procollagenase and the lysosomal enzymes elastase and cathepsin G were not different in the stunned and normal zone tissue, and thus exogenous sources of protease appear to be unlikely at the time of measurement (90-minute final reperfusion period) in this model of ischemia; and 4) dansyl chloride labeling of exposed N-terminal amino acid residues was 9% greater in the stunned than normal zone isolated collagen fibers. This latter result is compatible with greater in vivo cleavage of the stunned collagen matrix. Myocardial dysfunction was produced by a multiple coronary occlusion protocol, a procedure that has been demonstrated to produce myocardial dysfunction without any irreversible cellular damage.5,13,14 Thus, it appears that myocardial ischemia affects the extracellular compartment of the heart before irreversible structural changes in the cellular compartment. This differential sensitivity to ischemia should have important implications for cardiac enlargement and pathogenetic mechanisms in cardiomyopathy.

Expansion of the Stunned Myocardium

The extracellular collagen matrix of the heart interconnects myocyte to myocyte, myocyte to capillaries,
and myocyte to connective tissue. It also invests groups of myocytes (fascicles) and binds myocardial mural layers. Although its exact function has not been completely defined, its very nature is such that a coupling function in transmission of either active or passive force within the heart seems apparent. Thus, passive diastolic properties of muscle have been related to collagen content. A role of collagen in the active properties of muscle has also been suggested.

Forrester et al. and Theroux et al. found early increases in myocardial compliance during myocardial infarction. Eaton and Bulkey demonstrated early expansion of the infarcted myocardium, and this process was associated with a poorer prognosis. Infarct expansion appears to result in an increased incidence of myocardial aneurysm formation and rupture and in greater dysfunction. Factor et al. observed an absence of collagen in myocardial infarcts with ventricular rupture, suggesting a relation between the extracellular collagen matrix and infarct expansion. Ischemic myocardial expansion has been quantified by Weisman et al. and found to be predominantly due to cell slippage. The loss of intercellular and interlayer collagen matrix connections in the stunned myocardium may also result in cell slippage, with a secondary increase in regional dimensions. Zhao et al. found a significant uncoupling of the cell and muscle by constructing passive pressure-sarcomere length relations in the stunned and normal myocardium.

Whereas much emphasis has been paid to systolic dysfunction in stunned myocardium, diastolic abnormalities have received little attention. In the present study, a clear progressive relative increase in EDL occurred in the stunned myocardium and reached the maximum after the sixth reperfusion period. Geometrically, the 5–8% relative increase in linear dimension can translate into a threefold or 15–25% increase in regional volume. Both the normal and stunned regions experience the same diastolic pressure at any given time. Thus, the relative expansion index employed grossly accounts for variability in end-diastolic pressure during the course of the 4.5-hour protocol. Unlike systolic dysfunction, which demonstrates a rapid onset (Figure 1), diastolic expansion appears more gradual with a progressive increase in expansion until the sixth reperfusion period, where it plateaued (Figure 2). This may suggest different pathogenetic mechanisms for systolic and diastolic dysfunction in stunned myocardium. Nicklas et al. also found progressive expansion of the reperfused ischemic region using an identical repeated coronary artery occlusion protocol.

**Collagen Loss**

Only two studies have documented a quantitative decrease in cardiac collagen, and both of these studies examined infarcted myocardium. Cannon et al. found a 25% decrease in collagen at 24 hours with a subsequent increase at 72 hours in the infarcted rat myocardium. The latter increase was presumably the result of a rapid remodeling process. We have demonstrated significant

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**Figure 3.** Bar graph of collagenase activity (n=4). The total bar heights represent total collagenase activity (activation of procollagenase forms with aminophenylmercuric acetate [APMA]). The lower segmented bars represent collagenase activity without activation by APMA. Diagonally hatched bars represent the normal zone, and crosshatched bars represent stunned myocardium. Before complete activation of procollagenase forms, tissue collagenase activity was significantly greater in the stunned myocardium in all transmural layers compared with control (p<0.025). After activation with APMA, tissue collagenase activity was not different in the stunned and normal myocardium (total bar heights). This result suggests that exogenous sources of collagen do not appear to play a significant role in stunned myocardium, at least in the time frame of the protocol.

**Table 2. Tissue Proteolytic Activity**

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<th>Normal zone</th>
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<th>Stunned zone</th>
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<td></td>
<td>Endocardium</td>
<td>Midwall</td>
<td>Epicardium</td>
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<tr>
<td>Collagenase  (units/hr/100 mg)</td>
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<tr>
<td>APMA (−)</td>
<td>2.52±1.88</td>
<td>2.78±1.00</td>
<td>4.38±2.08</td>
<td>5.13±1.34*</td>
</tr>
<tr>
<td>APMA (+)</td>
<td>8.10±1.18</td>
<td>8.00±0.98</td>
<td>7.83±0.52</td>
<td>8.93±0.78</td>
</tr>
<tr>
<td>Elastase (munits/hr/100 mg)</td>
<td>8.08±1.52</td>
<td>8.13±1.00</td>
<td>7.98±1.16</td>
<td>7.65±1.64</td>
</tr>
<tr>
<td>Cathepsin G (munits/hr/100 mg)</td>
<td>3.03±0.50</td>
<td>2.60±0.42</td>
<td>2.65±0.80</td>
<td>3.18±0.28</td>
</tr>
</tbody>
</table>

Values represent mean±SD (n=4).

Collagenase: APMA (±), assay performed without (−) or with (+) addition of aminophenylmercuric acetate.

*p<0.025 compared with respective normal zone values. Values are expressed per 100 mg of protein.

Elastase and cathepsin G: There were no significant differences in activity in the stunned myocardium compared with the normal myocardium.
Mechanism of Collagen Degradation

Except for the neutrophil, collagenase is not stored intracellularly. Histologically, there does not appear to be an inflammatory response in the stunned myocardium when examined after the 90-minute final reperfusion period. Thus, increased collagenase activity requires either new synthesis or, more likely because of the rapid onset observed in the present study, extracellular activation of latent collagenase or procollagenase. Using immunohistochemical techniques, Montfort and Pérez-Tamayo observed collagenase antibody localization to collagen fibers in all organs including the heart. The findings suggested a "latent" form of collagenase intimately bound to its substrate. Collagenase inhibitors (tissue inhibitors of metalloproteinases) have also been identified in tissue. Activation of collagenase may result from extracellular cleavage of thezymogen form of the enzyme and/or by dissociation of the enzyme from a tissue inhibitor complex. In either case, no new synthesis of collagenase is required for rapid degradation of the matrix that occurs with ischemic insults of short duration. Compared with soluble collagen, the insoluble collagen found in the cardiac matrix takes 20-fold longer for collagenase to degrade. Woolley et al demonstrated that tissue collagenase alone is sufficient to completely degrade collagen (soluble and insoluble) without requiring other proteases.

The hearts for the protease assays were perfused with potassium phosphate buffer to remove serum inhibitors. α2-Macroglobulin, in particular, is a potent and irreversible inhibitor of collagenase. However, tissue inhibitors, as discussed above, were not removed. Unfortunately, in isolating collagenase from its tissue inhibitors, the purification process itself activates procollagenase to varying degrees. Thus, the present results using the crude homogenate should be interpreted accordingly. There was a significant increase in collagenase activity in the stunned myocardium compared with the normal region (Figure 3). Significant increases were found for all transmural layers, with an average 73.6% increase (p<0.025). The collagenase activity in the stunned epicardium was greater than the other layers but not statistically different. When procollagenase forms were activated by APMA, total collagenase activity was not different between stunned and normal regions (Figure 3). This result indicates that exogenous sources of collagenase, such as from neutrophils, are unlikely to be a significant factor in this model of ischemia for the time frame of the protocol. The overall results are compatible with activation of local sources of procollagenase as a result of the stunning process. Increased enzymatic activity in the stunned myocardium was also demonstrated indirectly by the dansylated collagen fiber results. Dansyl chloride reacts and labels exposed N-terminal amino acid residues on isolated collagen fibers from the stunned and normal zone. Increased dansyl labeling of collagen is highly suggestive for greater in vivo enzymatic cleavage of the fiber. Isolated fibers from the stunned myocardium had 9% greater dansyl labeling than the normal region (p<0.01). There was a tendency for greater dansyl labeling in the stunned epicardium and midwall compared with the stunned endocardium. It is possible that the transmural collagenase and dansylation patterns are related directly to the hydroxyproline results showing greater epicardial collagen loss. There were no differences in the lysosomal enzymes clastase or cathepsin G in the stunned and normal regions. There also were no transmural differences in these enzymes. These findings also make exogenous sources of degradative enzymes unlikely in the 90-minute recovery period time frame. However, it could be quite possible that neutrophil infiltration and increased lysosomal enzyme activity occur at a later time period.

Oxygen-derived free radicals have been implicated as a cause for the delayed functional recovery of the stunned myocardium. Bolli et al directly found the presence of free radicals in stunned myocardium using electron paramagnetic resonance. With regard to collagen and free radicals, Gallop et al demonstrated oxidation of collagen by hydrogen peroxide as a potential mechanism for its degradation. In that study, the strain was free of collagenase so that hydrogen peroxide, by itself, was presumably the sole cause of collagen degradation. Weiss et al demonstrated the activation of latent collagenase by a product of free radical metabolism. In that study, the activation of procollagenase by APMA was prevented by catalase. They tentatively identified the free radical product to be hypochlorous acid. Thus, oxygen-derived free radicals and their byproducts may affect collagen integrity either directly or indirectly via activation of collagenase. Caulfield and Wolkowicz demonstrated collagen degradation in isolated rat hearts after perfusion with exogenous disulfides. They speculated that endogenous colla-
genase was activated by the sulphydryl group of the disulfides. However, the strong oxidizing agents used may have activated collagenase via free radical formation.

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References
Collagen loss in the stunned myocardium.
R H Charney, S Takahashi, M Zhao, E H Sonnenblick and C Eng

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