Breakdown and Release of Myofilament Proteins During Ischemia and Ischemia/Reperfusion in Rat Hearts
Identification of Degradation Products and Effects on the pCa-Force Relation


Abstract—Our objective in experiments reported here was to identify myofilament proteins of rat hearts either lost or degraded by cardiac ischemia (15- or 60-minute duration) with and without 45 minutes of reperfusion. We correlated these changes with alterations in myofilament sensitivity to Ca$^{2+}$ and maximum force generation. Protein degradation and loss were assessed by high-performance liquid chromatography, SDS-PAGE, Western blotting analysis, and amino acid sequencing. Compared with nonischemic control hearts, bundles of skinned fibers from hearts subjected to ischemia alone demonstrated a decrease in maximum force generation and an increase in sensitivity to Ca$^{2+}$. These changes in function were increased with the duration of the ischemia and with reperfusion. With increasing duration of ischemia, there was an increased loss and degradation of myofibrillar α-actinin and troponin I (TnI) at its C-terminus. α-Actinin and TnI were most susceptible to ischemia, but with 60 minutes of ischemia/reperfusion, there was also degradation of myosin light chain-1 (MLC1) involving a clip of residues 1 to 19. The MLC1 degradation product was detected in the reperfusion effluent (along with troponin T, tropomyosin, and α-actinin) but not in the tissue with 60 minutes of ischemia with no reperfusion. Moreover, with ischemia the following proteins became associated with the myofibrils: GAPDH and proteins of the mitochondrial ATP synthase complex. Our results provide new evidence regarding the mechanism by which ischemia/reperfusion causes myocardial injury and support the hypothesis that an important element in the injury is altered activity and structure of the myofilaments. (Circ Res. 1998;82:261-271.)

Key Words: protein degradation ■ myocardial ischemia/reperfusion ■ myofilament ■ troponin I ■ α-actinin

Mechanisms responsible for cardiac dysfunction during myocardial ischemia and reperfusion appear likely to involve an altered response of the myofilaments to Ca$^{2+}$. Studies with isolated muscle preparations1 as well as perfused hearts2,3 demonstrated that the availability of Ca$^{2+}$ to trigger myofilament contraction is not a limiting factor during postischemic cardiac dysfunction. These studies showed that during ischemia, although myocardial function is depressed, the peak amplitude of intracellular Ca$^{2+}$ transients actually increases.

Although it is clear that accumulation of metabolites and protons during hypoxia and ischemia results in an acute change in myofilament response to Ca$^{2+}$, there is also evidence for persistent, but reversible, changes in myofilament response to Ca$^{2+}$. However, the exact nature of the myofilament changes appears complex and variable. For example, differences in maximum activity and sensitivity to Ca$^{2+}$ occurring in myofilaments isolated from control hearts and from hearts previously exposed to ischemia/reperfusion have been reported.5–7 The molecular mechanism may involve the breakdown and/or loss of myofilament proteins as well as an increased or new association of cellular cytoplasmic proteins with the myofilaments.5,9

Examination of the hearts and myofilaments from hearts stressed by ischemia/reperfusion indicates that both myofilament regulatory proteins9,10,11 and structural/cytoskeletal proteins12–13 are vulnerable to cleavage or loss. With mild ischemia/reperfusion, microtubules are substantially disrupted,13 and lesions occur within the tissues as a result of the loss of desmin, α-actinin, and spectrin.15,16 Immunohistochemical analysis of control and globally ischemic human left ventricle also demonstrated that a number of myofilament proteins, including actin, myosin, Tm, and TnT, may be lost from the tissue.14 In particular, TnI, a key thin-filament regulatory protein, has been shown to be proteolytically clipped during ischemia and ischemia/reperfusion.2,11 However, different laboratories have drawn varying conclusions about the extent and significance of TnI degradation.7,9,11

Received July 8, 1997; accepted October 31, 1997.
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The present study was undertaken to clarify the connection between myofilament dysfunction and changes at the protein level. Alterations were correlated to specific proteins with increasing degrees of ischemia, and it was determined whether these changes occurred during ischemia or on reperfusion. Using a variety of analytical approaches to identify myofilament, mitochondrial, and cytosolic proteins affected by ischemia with and without reperfusion, we report results that support the hypothesis that TnI is degraded during ischemia and stunning. We also demonstrate that α-actinin is lost from myofilaments even with mild ischemia and that severe ischemia results in the degradation of MLC1 and further degradation of TnI. The breakdown product of cardiac TnI is due to proteolysis at the C-terminus, whereas proteolysis of MLC1 occurs at its NH2-terminus. These changes in myofilament structure associated with ischemia/reperfusion appear to be important features of the depressed ability of the myofilaments to develop force and to their altered sensitivity to Ca2+. The present study is the first to simultaneously study both mild and severe ischemia with and without reperfusion and to correlate changes in myofilament function with changes at the protein level. Various parts of our results have been reported in abstract form.

Materials and Methods

Global Ischemic Model for Isolated Rat Hearts

Rats (250 to 350 g) were anesthetized with ether. The heart was excised and quickly placed in cold saline. The intact heart was placed in 2.5 mL saline within an airtight plastic bag for 60 minutes at 4°C (control) or 37°C. The use of a plastic bag follows a protocol used previously by our laboratory. It is a convenient and reproducible model that ensures temperature control and gives results that are similar to those obtained by global ischemia in isolated perfused hearts. The left ventricle was removed, and myofibrils were isolated according to the method of Rarick et al. A cocktail of protease inhibitors (50 μmol/L phenylmethylsulfonyl fluoride, 3.6 μmol/L leupeptin, and 2.1 μmol/L pepstatin A) was used at all steps of the isolation procedure. Isolated myofibrils were stored at −70°C until prepared for SDS-PAGE analysis.

Perfusion of Isolated Rat Hearts

Cardiac function was measured in a nonrecirculating Langendorff perfusion apparatus. Rats (250 to 350 g; average, 301 ± 33 g) were anesthetized with sodium pentobarbital (50 mg/kg) and injected with heparin (200 U) before the heart was excised. The hearts were quickly excised and placed in ice-cold saline. The aorta was cannulated, and the heart was perfused at a coronary flow of 14 mL/min with Krebs-Ringer bicarbonate buffer equilibrated with 95% O2/5% CO2 at 37°C. The Krebs-Ringer bicarbonate buffer consisted of 100 mmol/L sodium chloride, 4.74 mmol/L potassium chloride, 1.18 mmol/L potassium dihydrogen phosphate, 1.18 mmol/L magnesium sulfate, 1.15 mmol/L calcium chloride, 25 mmol/L sodium bicarbonate, 11.5 mmol/L glucose, 4.92 mmol/L pyruvate, and 5.39 mmol/L fumarate, pH 7.4. The hearts were paced at 360 bpm. All hearts were equilibrated with Krebs-Ringer bicarbonate buffer for 15 minutes before the experimental protocols described below. Hearts were subjected to either continuous flow for 45 minutes (control), 15 minutes of no-flow ischemia, or 60 minutes of no-flow ischemia with or without 45 minutes of reperfusion. No-flow ischemia was produced by wrapping the hearts in an impermeable plastic bag and submerging them in a water bath at 37°C. The perfusion pressure was measured from a side port in the perfusion apparatus, using a Statham P23 pressure transducer. Perfusion pressures were 61.5 ± 7.5 mm Hg during the 10-minute equilibration period and 78.8 ± 8.0 mm Hg after 45 minutes of perfusion. The pressure during reperfusion was 90.2 ± 17.3 and 133.5 ± 29.1 mm Hg after 15 minutes of ischemia and 60 minutes of ischemia, respectively.

Tissue and Effluent Sample Preparation

One-minute fractions of the effluent were collected at the end of the equilibration period. During reperfusion and the 45 minutes of perfusion (control), fractions were collected every minute for 10 minutes and then every 3 minutes for the remainder of the protocol. The fractions were frozen immediately at −70°C and then freeze-dried.

Skinned Fiber Bundle Experiments

Hearts removed from the Langendorff perfusion apparatus were immediately placed in cold relaxing buffer containing 0.1 mmol/L EGTA, 2 mmol/L Mg2+, 79.2 mmol/L potassium chloride, 5.0 mmol/L MgATP2−, 12 mmol/L creatine phosphate, and 20 mmol/L MOPS, pH 7.0 (ionic strength, 150 mmol/L), plus the protease inhibitor cocktail. Trabeculae were quickly dissected from the heart and placed in 50% (vol/vol) glycerol and relaxing buffer, protease inhibitor cocktail, and 10 mmol/L butanedione monoxime. The trabeculae were used in skinned fiber bundle experiments within a week. The remaining left ventricle was frozen on dry ice and stored at −70°C. The fiber bundles (≈100 μm in diameter) from each rat were glued to a force transducer at one end and to a fixed post attached to a micromanipulator. The fibers were skinned in relaxing buffer containing 10 IU/mL creatine kinase and 1% Triton X-100 for 30 minutes. The fibers were transferred to relaxing buffer containing 10 IU/mL creatine kinase, and the sarcomere lengths were set at 2.2 μm on the basis of the laser diffraction pattern. Isometric pCa-force relations were determined by bathing the skinned fiber bundles sequentially in solutions (10 mmol/L EGTA, 2 mmol/L Mg2+, 79.2 mmol/L potassium chloride, 5.0 mmol/L MgATP2−, 12 mmol/L creatine phosphate, 10 IU/mL creatine kinase, and 20 mmol/L MOPS, pH 7.0 [ionic strength, 150 mmol/L]) that contained increasing concentrations of calcium chloride to achieve pCa values from 8.0 to 4.5. All results are presented as mean ± SEM. Data were linearized using the Hill transformation, and the force/pCa relation was fitted to the Hill equation using nonlinear regression analysis to derive the pC50 and Hill coefficient. The total protein for each skinned fiber bundle was determined using the Lowry assay. At the same fiber bundle length, we found that relative tension expressed as force/cross-sectional area (average of controls, ≈50 mN/mm2) was similar to force/ng protein. This allowed an alternative comparison and analysis of relative tension generated by fiber bundles from different rat heart preparations with different treatments. In the figures, tension is normalized to the maximum tension of control preparations.

SDS-PAGE and Western Blot Analysis of Tissue and Effluent Samples

After Langendorff perfusion, left ventricular tissue samples were skinned in 50% (vol/vol) glycerol and relaxing buffer containing protease inhibitor cocktail. The myofibrils from the global ischemia model and the left ventricular tissue were homogenized in 160 mmol/L Tris, pH 8.8, plus the protease inhibitor cocktail. The protein content of the homogenate was determined using the Lowry assay. Homogenized samples were diluted 2-fold with sample buffer consisting of 2% SDS, 5 mmol/L Tris, pH 6.5, 20% sucrose, 0.025% bromophenol blue, and 1 mmol/L β-mercaptoethanol. Effluent
samples used for SDS-PAGE analysis were dialyzed against 1 mmol/L hydrochloric acid and 1 mmol/L β-mercaptoethanol with dialysis bags having a molecular weight cutoff of 6000. The samples were then freeze-dried and taken up into 50 μL of 160 mmol/L Tris, pH 8.0, plus the protease inhibitor cocktail and diluted 2-fold with sample buffer. Tissue samples (30 μg of total protein) and effluent samples (20 μg of total protein) were loaded on a 12.5% SDS polyacrylamide gel using a Hoeffer or Bio-Rad mini-gel apparatus. Gels were either stained with Coomassie blue or transferred to nitrocellulose (Bio-Rad) using a Hoeffer or Bio-Rad minigel apparatus. Gels were either stained with Coomassie blue or transferred to nitrocellulose (Bio-Rad) using a Hoeffer transfer system at 200 mA for 120 minutes or a Bio-Rad miniblot transfer system at 100 mA for 60 minutes. Proteins were quantified on the stained gel by densitometric scanning using an Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology). Western blot analysis was carried out according to the method described by Van Eyk et al. The monoclonal antibodies used were anti–TnI clone JLT-12 (Sigma Chemical Co), anti–α-actinin clone EA-53 (Sigma) or anti–α-actinin clone CH1 (Sigma), anti–sarcosmin actin clone SCS (Sigma), and anti–GAPDH (Cedarlane Laboratory Ltd). Three different anti–TnI antibodies were used in the present study: anti–TnI clone 3309, which recognizes amino acid residues 157 to 192 (provided by Dr J. Ladenson, Washington University, St Louis, Mo), anti–TnI clone 10F2 (Mab 10F2), which recognizes amino acid residues 189 to 199 (see epitope map; Fig 7), and anti–TnI peptide (P143T) 137 to 148 (Mab E2). The production of the anti–TnI peptide (P143T) 137 to 148 (equivalent to skeletal TnI residues 104 to 115) monoclonal antibodies, including Mab E2, has been described by Van Eyk et al. Mab E2 recognizes intact skeletal and cardiac TnI and cardiac TnI peptides containing amino acid residues 136 to 148 (data not shown). Epitope mapping of Mab 10F2 was carried out by 12% SDS-PAGE of intact cardiac TnI and various TnI fragments (2 to 5 μg) followed by Western blot analysis as outlined above. Bovine cardiac TnI was purified from the troponin complex by HPLC; recombinant cardiac TnI fragments 54 to 210, 1 to 188, and 1 to 199 were provided by Dr A. Martin (University Illinois at Chicago), and the synthetic skeletal TnI peptide 96 to 142, which is equivalent to the cardiac peptide residues 129 to 175, was prepared by solid-phase peptide synthesis.

Amino Acid Sequencing of Tissue and Effluent Samples
Tissue and effluent samples were prepared and electrophoresed by 12.5% SDS-PAGE as described above. The proteins were transferred onto PVDF (Bio-Rad) using 10 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid buffer at 100 mA for 55 minutes using a Bio-Rad minitransfer system. A Hewlett-Packard G1005A protein sequencer was used to sequence the initial amino acids of selected bands from the PVDF membrane using standard procedures (Alberta Peptide Institute).

HPLC Analysis of Effluent
The lyophillized effluent fractions were dissolved in 1 mL of water for every minute of perfusion. HPLC analysis of the effluent was performed on an analytical Zorbax C8 300SB reversed-phase column (4.6-mm internal diameter×250 mm, Chromagraphics Specialists Inc). The HPLC system consisted of a Hewlett-Packard series 1090 liquid chromatograph coupled to a Hewlett-Packard Vectra 486 166-MHz XM processor. The proteins were eluted using an AB solvent system. Buffer A was composed of 0.05% aqueous trifluoroacetic acid, and buffer B was composed of 0.05% trifluoroacetic acid in acetonitrile. The gradient consisted of an isocratic hold (100% buffer A) for 5 minutes followed by a 2% buffer B/min linear gradient at 1 mL/min. The proteins and protein fragments were monitored at 210 nm. The quantity of protein present in each effluent fraction was estimated by determining the area of the peak eluted at 23 minutes. We have previously shown that peak area is directly related to the quantity of the protein present. This method of quantification assumes that the same protein(s) is eluted at 23 minutes in the various effluent fractions from the different protocols.

Figure 1. The effect of reperfusion on the isometric force–pCa relation of skinned cardiac muscle bundles obtained from rat hearts that experienced increasing durations of ischemia. Isometric force was measured as a function of increasing Ca2+ concentrations for each skinned trabecular fiber bundle obtained from hearts that had undergone 15 minutes of equilibration followed by 45 minutes of perfusion (control, n=4, □), 60 minutes of ischemia (n=5, ▲), 15 minutes of ischemia followed by 45 minutes of reperfusion (n=4, △), 60 minutes of ischemia followed by 45 minutes of reperfusion (n=6, ○). Panel A shows tension plotted as a percentage of the maximum tension produced by the control fiber bundles. Panel B shows tension plotted relative to maximum force for each fiber bundle. The experimental protocols of the perfusion and skinned muscle fiber bundle analysis are described in “Materials and Methods.” Data are presented as mean±SEM for skinned fiber bundles obtained from four to six rat hearts for each experimental condition. Where the error bars are not shown, the standard error is smaller than the symbol.

Statistical Analyses
Statistical analysis was carried out on the data obtained from the force/pCa measurements and the densitometric measurements of the Coomassie blue–stained SDS-PAGE. Bartlett’s tests confirmed homogeneity of variances before one-way ANOVAs to determine differences between experimental groups. When significant F values were obtained in a given ANOVA, means were compared by Student-Newman-Keuls tests to isolate differences. For all analyses, a value of P≤.05 was accepted as significant. All data were presented as mean±SEM.

Results
Functional Experiments With Skinned Fiber Bundles
The first series of experiments involved determination of the effect of duration of ischemia and the effect of reperfusion on myofilament force development. Fig 1 shows the relation between pCa and isometric force developed by detergent-extracted fiber bundles obtained from rat hearts that had been perfused for 45 minutes in a control condition or reperfused for 0 or 45 minutes after ischemia lasting 15 or 60 minutes. The results depicted in Fig 1A demonstrate that preparations from hearts subjected to 15 minutes of ischemia developed the same maximum tension as control preparations (Table 1). However, preparations isolated from hearts exposed to 15 minutes of ischemia followed by 45 minutes of reperfusion demonstrated a substantial fall in Fmax. This fall in Fmax was similar to that
of preparations subjected to 60 minutes of ischemia. With 60 minutes of ischemia and 45 minutes of reperfusion, the Fmax of the skinned fiber bundles was even more depressed compared with the control values. There was also a leftward shift of the pCa-force relation: compare pCa\textsubscript{50} of 6.03 and 5.83 for skinned fiber bundles from hearts experiencing 60 minutes ischemia/45 minutes reperfusion and hearts perfused for 45 minutes (control), respectively (Fig 1B, Table 1). Although not as pronounced as in this example, preparations from the other groups of hearts also displayed greater sensitivity to Ca\textsuperscript{2+} than did control fiber bundles. These data indicate that (1) 60 minutes of ischemia alone will induce myofilament dysfunction, and (2) reperfusion or increasing duration of ischemia further aggravates the myofilament dysfunction.

### Analysis of Protein in the Reperfusion Effluent

The data depicted in Fig 1 indicate that functional alterations, which occur during both ischemia and ischemia/reperfusion, influence the response of the myofilament to both Ca\textsuperscript{2+} and Fmax. An important mechanism likely responsible for these effects is a loss or breakdown of myofilament proteins. We therefore determined the protein composition and any degradation products for the tissue samples of the left ventricle and the reperfusion effluent. The amount of protein present in the reperfusion effluent was monitored using reversed-phase HPLC to establish experimental conditions that represent reversible and irreversible damage. Reversed-phase HPLC separates proteins and peptides on the basis of hydrophobicity. The power of this approach is that, as shown previously, peak area is directly related to the amount of protein. Thus, one can estimate the amount of protein present in each fraction of reperfusion effluent by determining the area of a peak, which should represent one or more proteins present in the effluent. The appearance and subsequent disappearance of the peak in the effluent fractions during reperfusion provide a measure of the release kinetics as well as the relative amounts of protein present under different experimental conditions.

Samples of the reperfusion effluent were taken every minute for the first 10 minutes and every 3 minutes for the remaining reperfusion period, and Fig 2 shows representative protein elution profiles of these fractions for the 0-, 1-, and 10-minute samples after 60 (panel A) or 15 (panel B) minutes of ischemia. The inset shows an enlarged scale of the 0- to 1-minute fraction. Compared with 15 minutes of ischemia, the 60-minute ischemic episode resulted in release of many more different proteins and protein fragments. The results from HPLC analysis were confirmed by the 12.5% SDS-PAGE analysis of the combined samples after 60 minutes of ischemia (Fig 3A).

To quantify the protein in each fraction, we integrated the peak that is eluted at 23 minutes from the reversed-phase HPLC column in each of the reperfusion fractions collected after ischemia (Fig 2A and 2B). Potentially, this peak may arise from the absorbance of several proteins that are coeluted. The peak area from each fraction represents the amount of protein released on reperfusion during that time period. Fig 2C plots the area of the peak at 23 minutes for the fractions collected with respect to time. These data represent the release kinetics of these proteins. Several other peaks were quantified, and their release profiles were similar to that observed for the peak at 23 minutes (data not shown). In all cases, the majority of the protein was released from the heart within the initial 8 minutes of reperfusion. There was no protein present in the perfusate collected from control hearts. There is only a small amount of protein present in the reperfusion effluent after 15 minutes of ischemia compared with the amount of protein present after 60 minutes of ischemia. In fact, <5% of protein released from the heart after 60 minutes of ischemia is released and present in the effluent after 15 minutes of ischemia. The quantity of protein from the effluent (1 to 4 minutes) after 15 minutes of ischemia was such that even when several rats were combined, it was insufficient for SDS-PAGE or Western blot analysis. This suggests that there has been no breach in membrane integrity after 15 minutes of ischemia, a hallmark for irreversible

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**TABLE 1.** Functional and Protein Content of Left Ventricular Tissue From Rat Hearts That Have Undergone Ischemia and Ischemia/Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>L, min</th>
<th>RP, min</th>
<th>pCa\textsubscript{50}</th>
<th>Maximum Force, mg/mg</th>
<th>Tnl</th>
<th>α-Actinin</th>
<th>Tm</th>
<th>MLC1</th>
<th>OSC Protein MLC2</th>
<th>Quantity of Protein in Effluent, Relative Peak Area at 23 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>45</td>
<td>5.82±0.01</td>
<td>5190±72</td>
<td>0.28±0.05</td>
<td>0.16±0.01</td>
<td>0.29±0.05</td>
<td>0.13±0.03</td>
<td>ND</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>0</td>
<td>5.92±0.01*</td>
<td>5830±21†</td>
<td>0.28±0.02</td>
<td>0.17±0.02</td>
<td>0.24±0.01</td>
<td>0.13±0.01</td>
<td>ND</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>45</td>
<td>5.93±0.02*</td>
<td>2790±36†</td>
<td>0.37±0.02</td>
<td>0.10±0.04</td>
<td>0.24±0.01</td>
<td>0.18±0.01</td>
<td>0.05±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>0</td>
<td>5.86±0.02*</td>
<td>2860±52†</td>
<td>0.33±0.03</td>
<td>0.12±0.01</td>
<td>0.24±0.03</td>
<td>0.23±0.05</td>
<td>0.05±0.01</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>45</td>
<td>6.03±0.01*</td>
<td>1670±12†</td>
<td>0.51±0.07</td>
<td>0.10±0.02</td>
<td>0.29±0.02</td>
<td>0.30±0.04</td>
<td>0.09±0.02</td>
<td>0.19±0.05</td>
</tr>
</tbody>
</table>

ND indicates not detected; NA, not applicable (protocols where reperfusion was not done); L, ischemia; RP, reperfusion. All rat hearts underwent 15 minutes of equilibration before starting the experimental protocol. In the skinned fiber experiments, pCa\textsubscript{50} (−log concentration of Ca\textsuperscript{2+} required to induce half of the Ca\textsuperscript{2+}-dependent change in force) and maximum force (mg/mg) were determined from curve fitting the data in Fig 1. Force produced by a skinned fiber (mg) per total protein content of the corresponding skinned fiber (mg) with respect to changing Ca\textsuperscript{2+} concentrations is plotted. This quantifies the amount of force exhibited by each fiber taking into account the size of the fiber. The quantity of Tnl and α-actinin was determined by densitometry measurements (mean±SD) from a 12.5% SDS-polyacylamide gel (Fig 5). The quantity of protein present in the effluent was assigned a grading system (+++, most; ++, intermediate; and +, least) based on HPLC analysis of effluent samples (Fig 2). Group 1 reflects control conditions (0 minutes of ischemia followed by 45 minutes of perfusion). The Student-Newman-Keuls method was used to compare all of the different ischemia and ischemia/reperfusion data with control data.

*P<0.05 and †P=NS vs control (group 1) data.

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damage. It is well established that myofilament proteins and cytoplasmic proteins, such as creatine kinase, are lost from hearts exhibiting myocyte necrosis, loss of membrane integrity, and irreversible damage to the cell. This is the basis for the serum diagnostic for myocardial infarction (for review, see References 28 and 29). In this experimental isolated heart model, the release of myofilament proteins and their detection in the reperfusion effluent would suggest that irreversible damage had occurred at 60 and not 15 minutes of ischemia. Therefore, the change from reversible to irreversible damage occurs after 15 minutes of ischemia.

Analysis of Proteins in Left Ventricular Tissue Samples

To determine whether the functional changes in skinned fiber bundles from hearts perfused under a variety of conditions are correlated with degradation or loss of myofilament proteins, SDS-PAGE, Western blot analysis, and amino acid sequencing were used to analyze the state of the myofilament proteins. Fig 4 shows representative 12.5% SDS-PAGE and Western blot analysis of left ventricular tissue samples from hearts subjected to 45 minutes of control perfusion, 15 minutes of ischemia followed by 45 minutes of reperfusion, 60 minutes of ischemia, or 60 minutes of ischemia followed by 45 minutes of reperfusion. Western blots using antibodies against α-actinin, Tin, and TinT proteins that are present in the reperfusion effluent after 60 minutes of ischemia (Fig 3), revealed that these proteins were not degraded. MLC2 was also present in the tissue as an intact protein. Thus, loss of these proteins from the cell was not the result of degradation. Data shown in Figs 3 and
4 indicate that this does not appear to be the case for TnI, MLC1, and α-actinin.

As determined by densitometric measurement of protein profiles on 12.5% gels of skinned tissue samples (Fig 5A and 5B, Table 1), there was a 37% drop in the amount of protein migrating at the same mobility as α-actinin. This loss of α-actinin occurred with 15-minute ischemia/45-minute reperfusion, 60-minute ischemia, and 60-minute ischemia/45-minute reperfusion. No loss of protein occurred with 15-minute ischemia alone (data not shown), indicating that α-actinin loss from the tissue was augmented by reperfusion. Quantification of the amount of α-actinin in the tissue samples using Western blot analysis (Fig 4) indicated that densitometric analysis (Fig 5, Table 1) overestimated the amount of protein. It is therefore likely that there was another protein or fragment co-migrating with α-actinin in the samples from the ischemic/reperfused hearts. We recognize that our conclusion is subject to potential inaccuracies in the quantification of the protein using Western blot analysis. However, α-actinin was also lost from isolated myofibrils prepared from globally ischemic rat hearts (Fig 6).

Using 10% SDS-PAGE, we could detect degradation of α-actinin in the tissue from hearts exposed to 60 minutes of ischemia (Fig 4g). However, with reperfusion, no degradation product could be detected in the small amount of α-actinin left in the tissue or in the reperfusion effluent. Previous failures to detect loss of α-actinin by SDS-PAGE analysis during ischemia/reperfusion injury could be due to reperfusion of the tissue, preparation of the tissue samples (not skinned), and use of densitometry of Coomassie blue–stained gel. In our case, densitometry of Coomassie blue–stained gels detected only a small percentage of the loss of α-actinin compared with that observed by Western blot analysis (compare Fig 5B or Table 1 with Fig 4). In unskinned tissue samples, intact and degraded α-actinin could not be differentiated using 12.5% SDS-PAGE (data not shown). However, when the muscle cell membranes were removed (1% Triton X-100 and/or 50% glycerol) before preparing the samples for gel electrophoresis, the susceptibility of α-actinin to loss and degradation became apparent by Western blot analysis (Fig 4). Isolated myofibrils from globally ischemic rat hearts demonstrated the same loss of α-actinin compared with control myofibrils (Fig 6).

TnI degradation was observed by Western blot analysis in tissue obtained from 60-minute ischemic and 15- and 60-minute ischemic/reperfused hearts (Fig 4). TnI degradation appears to be a controlled and specific digestion that produced a single degradation product after 15-minute ischemia/45-minute reperfusion or 60-minute ischemia alone. However, there were at least two degradation products after 60 minutes of ischemia and 45 minutes of reperfusion. TnI degradation was also observed in isolated myofibrils from globally ischemic hearts (Fig 6). In both cases the TnI degradation product(s) comigrated on a 12.5% SDS-polyacrylamide gel at the same mobility as the 15-minute reperfusion, 60-minute ischemia, and 60-minute ischemia/45-minute reperfusion. No loss of protein occurred with 15-minute ischemia alone (data not shown), indicating that α-actinin loss from the tissue was augmented by reperfusion. Quantification of the amount of α-actinin in the tissue samples using Western blot analysis (Fig 4) indicated that densitometric analysis (Fig 5, Table 1) overestimated the amount of protein. It is therefore likely that there was another protein or fragment co-migrating with α-actinin in the samples from the ischemic/reperfused hearts. We recognize that our conclusion is subject to potential inaccuracies in the quantification of the protein using Western blot analysis. However, α-actinin was also lost from isolated myofibrils prepared from globally ischemic rat hearts (Fig 6).

Using 10% SDS-PAGE, we could detect degradation of α-actinin in the tissue from hearts exposed to 60 minutes of ischemia (Fig 4g). However, with reperfusion, no degradation product could be detected in the small amount of α-actinin left in the tissue or in the reperfusion effluent. Previous failures to detect loss of α-actinin by SDS-PAGE analysis during ischemia/reperfusion injury could be due to reperfusion of the tissue, preparation of the tissue samples (not skinned), and use of densitometry of Coomassie blue–stained gel. In our case, densitometry of Coomassie blue–stained gels detected only a small percentage of the loss of α-actinin compared with that observed by Western blot analysis (compare Fig 5B or Table 1 with Fig 4). In unskinned tissue samples, intact and degraded α-actinin could not be differentiated using 12.5% SDS-PAGE (data not shown). However, when the muscle cell membranes were removed (1% Triton X-100 and/or 50% glycerol) before preparing the samples for gel electrophoresis, the susceptibility of α-actinin to loss and degradation became apparent by Western blot analysis (Fig 4). Isolated myofibrils from globally ischemic rat hearts demonstrated the same loss of α-actinin compared with control myofibrils (Fig 6).
location as intact MLC1. This affected the ability to quantify the amount of protein by SDS-PAGE densitometry under the band that migrated at the position of intact MLC. In fact, the amount of protein detected at that molecular weight was greater in ischemic than in control tissue (Fig 5B, Table 1). This is most likely due to the combination of intact MLC (at reduced levels as a result of degradation and loss) and the TnI degradation product. By densitometric analysis of the 12.5% SDS-PAGE (Fig 5B, Table 1), both MLC1 and TnI increased with 60 minutes of ischemia followed by 45 minutes of reperfusion. Densitometric analysis of detergent extracted muscle tissue from hearts subjected to 15 or 60 minutes of ischemia followed by 45 minutes of reperfusion revealed an increase in protein migrating with the mobility of TnI (Table 1, Fig 5C). This reemphasizes the difficulty in detecting and quantifying changes in the myofilament proteins using SDS-PAGE and densitometry, which may lead to erroneous conclusions. However, since two anti-TnI monoclonal antibodies (E2 and 3309) recognizing different epitope sequences within cardiac TnI are able to detect the TnI degradation products, there can be no mistake as to the identity of the degradation product.

To characterize the TnI degradation product, N-terminal amino acid sequencing was carried out (Table 2). Since the N-termini of both TnI and MLC1 are acetylated, amino acid sequencing would yield the TnI fragment only if degradation occurred within the N-terminus. Because sequencing only detected low levels of unacetylated MLC1, neither sequencing nor the quantity of protein available for sequencing was limiting. This indicates that the N-terminus of TnI fragment is acetylated and that the cleavage has occurred at the C-terminus. Furthermore, we determined the epitope of the anti-TnI antibody (Mab 10F2), which binds strongly to intact TnI and only weakly to the degradation product (Fig 7A). Mab 10F2 epitope lies within amino acid residues 188 to 199 (Fig 7B), indicating that the TnI degradation product does not contain all or part of this sequence.
Degradation of MLC1 was detected in myofibrils isolated from globally ischemic hearts using Western blot analysis (Fig 6). Amino acid sequencing of this band on PVDF membrane identified the fragment to be residues 20 to 199 (Table 2). What is interesting is that the degradation product was not seen in any of the ischemic tissue samples (Fig 4) but that it was found in the reperfusion effluent collected after 60 minutes of ischemia (Fig 2). This suggests that MLC1 degradation occurs with severe ischemia as seen in the globally ischemic hearts. The ischemic injury to the perfused heart after 60 minutes of ischemia appears not to be as severe, inasmuch as there was no MLC1 degradation. However, our data indicate that reperfusion augmented the damage already in evidence after 60 minutes of ischemia and was sufficient to result in the degradation of MLC1.

Nonmyofilament proteins associated with the ischemic and ischemic/reperfused tissues were also found. As previously reported,8,9 the cytoplasmic protein, GAPDH, was present in all ischemic tissue samples (data not shown). As well, amino acid sequencing from the PVDF membrane of the 12.5% SDS-PAGE of the 60-minute ischemia/45-minute reperfused tissue sample identified two mitochondrial proteins present in the prepared tissue sample (Table 2). These proteins were identified as the ATP synthase γ chain and the ATP synthase OSC protein. These proteins migrated in two bands, which became more prominent in the 60-minute ischemia/45-minute reperfusion sample than in the control tissue sample (Fig 5A, Table 1). However, the other proteins, which have their N-terminus blocked (and thus cannot be detected by amino acid sequencing) may also contribute to this increase in protein at these bands. It has been reported that α/β crystallin migrates at the same location as the ATP synthase OSC protein.8,9 Thus, the increase in the protein migrating at this molecular weight could result from contributions from both of these proteins and, possibly, from other unidentified protein(s).

Discussion

Our data support the hypothesis that sustained changes in myocardial function associated with ischemia and reperfusion involve an altered response of the myofilaments to Ca\(^{2+}\). Important new findings presented in the present study are: (1) demonstration of the relatively high susceptibility of TnI breakdown and α-actinin loss to ischemia/reperfusion, (2) the effects on the myofilaments’ progress with increasing severity of ischemia/reperfusion, such that there is MLC1 degradation and an increase in the number of TnI degradation products, and (3) the identification of the TnI and MLC1 degradation products. These results confirm and extend previous data indicating that proteins not normally associated with myofilaments are retained and apparently bound by the myofilaments even after the skinning procedure. These changes in myofilament structure provide a plausible mechanism for the functional changes associated with brief ischemic episodes and for the more severe changes in function associated with prolonged ischemia.

Functional Changes Induced in Myofilaments Stressed by Ischemia/Reperfusion

There is a spectrum of results regarding the extent and nature of myofilament dysfunction associated with mild and severe ischemia/reperfusion injury. This is most likely due to variations in the species studied, the duration of the ischemia, and whether in situ or isolated heart models were studied. Nevertheless, there is general agreement that myofilament response to Ca\(^{2+}\) is altered. This study is the first to examine simultaneously both mild (15-minute) and severe (60-minute) ischemia with and without reperfusion using the same experimental animal model. Compared with control preparations, there was a reduction in maximum tension generated by myofilaments of isolated rat hearts subjected to 15 minutes of ischemia with reperfusion. These myofilaments were slightly more sensitive to Ca\(^{2+}\) (Table 1, Fig 1). Although Gao et al,7,30 who also studied the effects of ischemia on rat heart preparations, reported a decrease in maximum tension, there was a small decrease in sensitivity to Ca\(^{2+}\). Skinned myocytes isolated from pig hearts after 45 minutes of low-flow in situ ischemia with reperfusion (stunning) also showed a decrease in Ca\(^{2+}\) sensitivity but no change in maximum force generation compared with the control myocytes.6,31 This laboratory62 has also indicated that although stunning had little effect on the steady-state force, it may alter crossbridge kinetics, shifting it toward force-generating states. However, skinned myocytes by the nature of their isolation represent a selective population of cells compared with skinned trabecular muscle fiber bundles, which are inclusive of the populations represented in the left ventricle at the time of experiment; thus, these two experimental models may show differences in their response to Ca\(^{2+}\). Interestingly, as was the case in the present study using skinned trabecular fiber bundles (Fig 1, Table 1), Miller et al,21 using myocytes isolated from stunned in situ hearts, showed that the major functional damage occurs on reperfusion.

With more severe ischemia (60-minute ischemia/45-minute reperfusion), the present study showed that the isolated skinned trabecular fiber bundles display a relatively large decrease in maximum force and a significant increase in Ca\(^{2+}\)
sensitivity compared with control fiber bundles (Table 1, Fig 1). Arner et al also found an increase in Ca\(^{2+}\) sensitivity with skinned trabecular fiber bundles obtained from in situ global ischemia in dog hearts. However, Dietrich et al.\(^{15}\) found little or no change in Ca\(^{2+}\) sensitivity or maximum force in skinned trabecular fiber bundles obtained from isolated rat hearts that underwent 40-minute ischemia/30-minute reperfusion.

These variations in the effects of ischemia and reperfusion on myofilament function may have been due to the influence of small variations in ischemic duration that gave rise to large effects on the severity or nature of the injury that occurs. For example, Gao et al.\(^{60}\) applied 20 minutes of no-flow ischemia, which produced a different effect than the 15 minutes of ischemia that was used in the present experiments. With 15 minutes of ischemia, there was little release of protein and no TnT release from the heart on reperfusion. However, Yahara et al.\(^{34}\) showed that TnT is released from isolated hearts minutes of ischemia, there was little release of protein and no TnI degradation product was not confirmed in a subsequent study, 9 we have identified the TnI degradation as an important feature of ischemic damage. We have identified the TnI degradation product using two different anti-TnI antibodies (Mab E2 and 3309) that recognize different amino acid sequences of TnI. This eliminates the possibility of nonspecific cross-reactivity with proteins other than TnI.

An important aspect of the results presented here is that the degradation product of TnI, which we believe is responsible in part for the functional changes, remains bound to the myofilaments under severe conditions, such as detergent treatment. In contrast to the case with α-actinin, MLC1 (as well as MLC1 fragment 20 to 199), Tm, and TnT, all of which appeared in the reperfusion effluent after 60 minutes of ischemia, neither TnI nor TnI degradation products were detected in the same effluent. This indicates that the TnI degradation products are likely to have a role in the development of the myofilament dysfunction, especially since degradation progresses from one to two degradation products as the time of ischemia increases.

The tightly bound degradation products arise from the hydrolysis at the C-terminus of TnI. This proposal is based on our inability to sequence the product, which is undoubtedly small differences in the quantities of either intact troponin (complex of TnT, TnI, and TnC), TnI, TnC, or MLC2 lost from the myofilaments could dramatically affect myofilament response to Ca\(^{2+}\).\(^{35-39}\) Previous data from one of our laboratories\(^{39}\) indicated that changes in the quantity of TnI bound to the thin filament produce dramatic shifts in the pCa-force relation to either higher or lower pCa\(_{50}\) values (pCa\(_{50}\) is the−log calcium concentration required to induce 50% of the maximum Ca\(^{2+}\)-dependent force). Moreover, myofilament control mechanisms may be quite sensitive to effects of proteolytic products. In fact, the addition of as little as four N-terminal peptides of MLC1 per thin filament can dramatically change the response of the myofilaments to Ca\(^{2+}\).\(^{19}\) Although there are conflicting reports on the nature and extent of myofilament dysfunction with different degrees of ischemia/reperfusion injury, the present study is the first to correlate the severity of ischemia/reperfusion injury with both an increase in myofilament dysfunction and the extent of myofilament protein degradation or loss.

Changes in Myofilament Proteins Induced by Ischemia/Reperfusion

Earlier studies have also implicated troponin, or one of its subunits, as a site for lesions associated with myocardial ischemia/reperfusion–induced injury. In a study of dog hearts, Toyo-Oka and Ross\(^{90}\) reported a loss of TnI from myofilaments isolated from myocardial tissue made ischemic in situ. A loss of TnI was also suggested by the results of Andres et al.\(^{60}\) on the basis of the response to isoproterenol of isolated rat hearts that experienced 20 minutes of ischemia followed by reperfusion. These myofilaments demonstrated an increase in Ca\(^{2+}\) sensitivity compared with preparations from control hearts. We previously provided evidence that myofilaments from isolated rat hearts made globally ischemic for 60 minutes contain degradation products of both TnI and TnT.\(^{11}\) Although a TnI degradation product was not confirmed in a subsequent study,\(^{7}\) we have shown in the present study that TnI is degraded during global ischemia (Fig 6). Furthermore, the recent results reported by Gao et al.,\(^{2}\) together with data in the present study, confirm our earlier conclusion that TnI degradation is an important feature of ischemic damage. We have identified the TnI degradation product using two different anti-TnI antibodies (Mab E2 and 3309) that recognize different amino acid sequences of TnI. This eliminates the possibility of nonspecific cross-reactivity with proteins other than TnI.

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Reduced actin-myosin affinity and myosin ATPase activity have been reported for cardiac myosin containing an MLC1 fragment that was proteolysed at its N-terminus. This suggested that MLC1 fragment 20 to 199 could alter function. However, since the MLC fragment is released from the cell during severe ischemia (Fig 3), it would make only a small contribution toward the myofilament dysfunction observed with 60 minutes of ischemia and 45 minutes of reperfusion. Most likely, the loss of function under these severe conditions is due to the massive loss of cell membrane integrity and the release of cytoplasm and many myofilament proteins from the cell.

Our results extend previous data showing that cellular proteins not normally associated with contractile proteins bind to myofilaments during ischemia/reperfusion. GAPDH, a cytosolic protein, seems to associate with the actin filament under ischemic conditions. It has been previously reported that another cytoplasmic protein, α/β crystallin, also may associate with the myofilament under ischemic or anoxic conditions. We have confirmed the presence of these proteins in tissue obtained from hearts that experienced 15 or 60 minutes of ischemia/reperfusion as well as in the reperfusion effluent after 60 minutes of ischemia, but not in control hearts. In addition to these proteins, we detected ATP synthase γ chain and OSC proteins. These proteins are localized on the cytoplasmic side of the plasma membrane of the ATPase synthase complex located in the inner membrane of the mitochondria. Earlier work showed that when anoxic cultured heart cells are reoxygenated, there is a release of proteins situated on the outer membrane of the mitochondria. Blood of patients with myocardial infarction show release of mitochondrial proteins. Our data shed new light on this release and indicate that two effects may result: a loss of ATP synthetic capacity and a potential alteration in the myofilaments as these products associate with the contractile proteins. Substantiation of this latter speculation requires systematic investigation of the effects of the ATP synthase proteins on myofilament activity and regulation.

Mechanisms for Changes in Myofilament Proteins

Degradation and loss of some of these proteins may be attributable to Ca$^{2+}$-dependent proteases, which have been proposed to be activated during the Ca$^{2+}$ overload during late ischemia and early reperfusion. One such Ca$^{2+}$-dependent protease thought to play a role in ischemia is calpain, which is localized near the Z line. In vitro, α-actinin, spectrin, desmin, TnI, and TnT are susceptible to degradation by calpain. Functional protection occurs by addition of a calpain inhibitor during reperfusion of an isolated heart following ischemia. The addition of calpain to skinned muscle fibers causes a decrease in rigor tension with a concurrent loss of a 95-kD protein. This protein is probably α-actinin because of its molecular weight and because the addition of calpain to myofibrils or skinned muscle fibers results in the loss of the Z line (which is primarily composed of α-actinin). In addition to the loss of α-actinin, Gao et al recently showed that addition of calpain to skinned muscle fibers yields the same or similar TnI degradation product as mild ischemia/reperfusion. Thus, exogenous calpain mimics the alterations observed in the myofilament proteins, ie, loss of α-actinin and degradation of TnI at the C-terminus during mild ischemia. Whether other proteases, such as mekratin, are involved remains an open question.

The present study clearly shows that the contractile proteins TnI and α-actinin are highly susceptible to progressive damage during ischemia and on reperfusion. The alterations in TnI and α-actinin provide a plausible mechanism for the dysfunction observed in skinned trabecular muscle fibers from isolated hearts that have experienced ischemia with and without reperfusion. Understanding the consequences of these changes to the myofilament proteins is critical for the development of new diagnostic strategies and new therapies for the protection and treatment of ischemia/reperfusion injury.

Acknowledgments

This study was supported by the Ontario Heart and Stroke Foundation (Dr Van Eyk), a VA merit review grant (Dr Law), the Alberta Heart and Stroke Foundation (Dr Hodges), and National Institutes of Health grants HL-22231 and HL-49934 (Dr Solaro).

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Circ Res. 1998;82:261-271
doi: 10.1161/01.RES.82.2.261

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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