Troponin I Degradation and Covalent Complex Formation Accompanies Myocardial Ischemia/Reperfusion Injury

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Abstract—Selective troponin I (TnI) modification has been demonstrated to be in part responsible for the contractile dysfunction observed with myocardial ischemia/reperfusion injury. We have isolated and characterized modified TnI products in isolated rat hearts after 0, 15, or 60 minutes of ischemia followed by 45 minutes of reperfusion using affinity chromatography with cardiac troponin C (TnC) and an anti-TnI antibody, immunological mapping, reversed-phase high-performance liquid chromatography, and mass spectrometry. Rat cardiac TnI becomes progressively degraded from 210 amino acid residues to residues 1–193, 63–193, and 73–193 with increased severity of injury. Degradation is accompanied by formation of covalent complexes between TnI 1–193 and, respectively, TnC residues 1–94 and troponin T (TnT) residues 191–298. The covalent complexes are likely a result of isopeptide bond formation between lysine 193 of TnI and glutamine 191 of TnT by the cross-linking enzyme transglutaminase. With severe ischemia, cellular necrosis results in specific release of TnI 1–193 into the reperfusion effluent and TnT degradation in the myocardium (25-, 27-, and 33-kDa products). Two-dimensional electrophoresis demonstrated that phosphorylation of TnI prevents ischemia-induced degradation. This study characterized the modified TnI products in isolated rat hearts reperfused after a brief or severe period of ischemia, revealing the progressive nature of TnI degradation, changes in phosphorylation, and covalent complexes with ischemia/reperfusion injury. Finally, we propose a model for ischemia/reperfusion injury in which the extent of proteolytic and transglutaminase activities ultimately determines whether apoptosis or necrosis is achieved. (Circ Res. 1999;84:9-20.)

Key Words: protein degradation ■ myocardial ischemia ■ myofilament ■ troponin I ■ transglutaminase

Myocardial ischemia/reperfusion presents a spectrum of injury, from reversible damage with mild ischemia, to irreversible cellular necrosis with severe ischemia. Identification of proteins modified during ischemia/reperfusion is fundamental to our understanding of the pathological processes involved. Several laboratories have shown that myofilament proteins, including the troponin complex, are specifically degraded or modified under ischemia/reperfusion.1–9 However, the modified troponin products produced by ischemia/reperfusion injury are not yet identified or characterized.

The troponin complex is the regulatory element of the myofilament, which mediates the calcium dependence of muscle contraction in both cardiac and skeletal muscle. Its 3 components, troponin I (TnI), troponin C (TnC), and troponin T (TnT), interact with each other and other thin filament proteins (eg, actin and tropomyosin) through both calcium-dependent and -independent associations.10,11 Calcium binding to TnC at the N-terminal regulatory site produces a conformational change in TnC and movement of TnI away from actin-tropomyosin. Cardiac (c) TnI has a 30 to 32 amino acid N-terminal extension compared with skeletal (s) TnI, which decreases the affinity of TnI for TnC when phosphorylated by protein kinase A (PKA).12,13 The calcium-dependent movement of TnI away from actin reveals a tropomyosin binding site, which results in movement of tropomyosin away from high-affinity myosin binding sites on actin.10,11

Several studies have demonstrated selective TnI degradation1–7 under ischemia/reperfusion injury, as well as changes in TnT immunoreactivity.8,9 However, each study uses different ischemia/reperfusion periods and different models of ischemic damage (eg, coronary artery occlusion, global ischemia, Langendorff perfusion, and various other combinations).

Myofilament regulatory proteins1–4 and structural/cytoskeletal proteins5,6,14 have both been shown to be susceptible to cleavage or loss under myocardial ischemia/reperfusion. Mild ischemia/reperfusion has been shown to disrupt microtubules14 and produces lesions resulting from loss of desmin, α-actinin, and spectrin.15,16 The contractile proteins actin, myosin, tropomyosin, and TnT are lost from globally ischemic human left ventricle tissue.6 In particular, TnI has been shown to be proteolytically cleaved during ischemia and ischemia/reperfusion.2,3 Different groups have drawn varying conclusions about the extent and functional importance of...
TnI degradation \(^2,^3,^7\); however, it is generally agreed that TnI degradation occurs and correlates to contractile dysfunction.

In this study, we have isolated and characterized the modifications to TnI in both mildly and severely ischemic isolated perfused rat hearts. Through the use of tricine-SDS-PAGE (T-PAGE) and Western blot analysis, we have shown that TnI degradation is a progressive process. Increases in the severity of the ischemic insult lead to more extensive degradation of TnI and preferential release of a TnI degradation product into the reperfusion effluent. Strong indirect evidence has shown that the calcium-dependent protease calpain is associated with rcTnI \(^1–188\), but it does not bind rcTnI \(^129–175\). MBP AM-IN was mapped elsewhere. The epitopes of anti-TnI MAb 3I-35 (Spectral Diagnostics) and anti-TnC MAb (Sigma) have not been identified.

**Materials and Methods**

**Langendorff Perfusion of Isolated Rat Hearts**

We adapted this technique from a previously published protocol.\(^1\) Male Sprague-Dawley rats (250 to 275 g) were anesthetized with sodium pentobarbital (\(100 \text{ mg/kg}\)) and heparinized via the inferior vena cava (\(50 \text{ mg/kg}\)). Hearts were quickly excised and placed in ice-cold saline. The aorta was cannulated on a 1.6-mm glass cannula (Radnoti Glass Inc) and perfused in a nonrecirculating apparatus with heart chamber (Radnoti Glass Inc) at 14 mL/min with Krebs-Ringer bicarbonate buffer (\([\text{in mmol/L}]\) sodium chloride 100, potassium chloride 4.74, potassium dihydrogen phosphate 1.18, magnesium sulfate 1.18, calcium chloride 1.15, sodium dihydrogen carbonate 25, glucose 11.5, sodium pyruvate 4.92, and sodium fumarate 5.39, pH 7.4) equilibrated with 95% \(\text{O}_2/5\% \text{CO}_2\) at 37°C. The right atrium was fitted with electrodes, and the hearts were paced at 360 bpm. Hearts were subjected to 15 minutes of equilibration followed by 0, 15, or 60 minutes of ischemia (no flow and no stimulation) and 45 minutes of reperfusion (0/45, 15/45, and 60/45, respectively). The effluent from the initial 10 minutes of reperfusion was collected, lyophilized, and stored at –20°C until analysis. We have previously shown that the majority of proteins are released in the first 10 minutes of reperfusion.\(^1\) The left ventricle was immediately frozen in liquid nitrogen and stored at –70°C until analysis.

**Isolation and Identification of Modified TnI Products**

**Tissue Homogenization**

Left ventricles (\(n=3\)) were homogenized together in 20 mmol/L Tris-HCl, pH 7.4, 6 mol/L urea, and 200 mmol/L potassium chloride, with a protease inhibitor cocktail (3.6 \(\mu\text{mol/L}\) leupeptin, 2.1 \(\mu\text{mol/L}\) pepstatin A, and 50 \(\mu\text{mol/L}\) phenylmethylsulfonylfluoride) on ice. Aliquots of tissue homogenates for electrophoresis were stored at –20°C until use. Remaining tissue homogenates for affinity chromatography were dialyzed at 4°C against 2 mol/L urea, 1 mol/L potassium chloride, 1 mol/L DTT, and 200 mmol/L Tris-HCl, pH 7.4, with the protease inhibitor cocktail, followed by sequential dialysis against 1 mmol/L DTT, 0.1 mmol/L EDTA, 20 mmol/L Tris-HCl, pH 7.4, and protease inhibitor cocktail, with decreasing concentration of potassium chloride from 1 mol/L to 500 and 200 mmol/L (2 changes). In the final 2 dialysis steps (200 mmol/L potassium chloride), DTT was not present, and tissue samples were maintained in reduced form with nitrogen gas bubbled through the dialysis buffer. Samples were centrifuged at 15 000 rpm for 10 minutes at 4°C, and the supernatant was used for further analysis.

Total protein concentration of tissue homogenate supernatants and dialysis buffer was determined according to Lowry et al.\(^20\) Protein concentration of tissue homogenate supernatants and dialysis buffer was determined according to Lowry et al.\(^20\) Bovine cTnI, cTnC, and cTnT and rabbit sTnC were isolated and purified as published by Ingraham and Hodges.\(^21\)

**Affinity Chromatography**

Rabbit sTnC was cross-linked to 3M Emphaze resin (Pierce) according to the manufacturer’s protocol. The anti-TnI monoclonal
antibody (MAb) 8I-7 (Spectral Diagnostics, Figure 1) was cross-linked to cyanogen bromide Sepharose (Pharmacia) according to the manufacturer’s protocol. Both affinity columns were equilibrated in buffer A consisting of 20 mmol/L Tris-HCl, pH 7.4, 50 mmol/L potassium chloride, and 1 mmol/L calcium chloride with the protease inhibitor cocktail. Left ventricle tissue supernatants were loaded onto either column (~10 mg), and the column was washed with 10 volumes of buffer A. Bound proteins were eluted with 65 mmol/L glycine-HCl, pH 3.2. Fractions (1 mL) were collected into tubes containing 100 μL of 0.66 mol/L MOPS, pH 8.0, to neutralize the pH to 7.5. After lyophilization, fraction volumes were resuspended in 0.5% aqueous trifluoroacetic acid and analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC), using an analytical Zorbax C8 300SB RP column (4.6 mm internal diameter × 250 mm, Chromatographic Specialties Inc.). The HPLC system consisted of a Varian (Mississauga, Canada) 9100 autosampler, 9012 solvent delivery system, and 9065 diode ray detector. The proteins were eluted using an AB solvent system, in which solvent A was composed of 0.05% aqueous trifluoroacetic acid and solvent B was composed of 0.05% trifluoroacetic acid in acetonitrile. The AB gradient consisted of an isocratic hold (100% solvent A) for 5 minutes followed by a 2% solvent B/min linear gradient at a flow rate of 1 mL/min. The peaks were collected, lyophilized, and analyzed by mass spectrometry, Western blotting, and amino acid microsequencing (Alberta Peptide Institute, University of Alberta, Edmonton, Canada).

Gel Electrophoresis and Western Blotting

Tissue homogenates, effluent samples, and the peaks collected from RP-HPLC analysis of anti-TnI MAb affinity chromatography-bound fractions were separated by 12.5% SDS-PAGE, or by T-PAGE22 in the presence of 6 mol/L urea, using the Mini-gel system (Bio-Rad), T-PAGE was performed with a 10%T (total acrylamide concentration), 3%C (concentration of bis-acrylamide) resolving gel and 4%T, 3%C stacking gel containing 6 mol/L urea, 0.1% SDS, and 0.1 mol/L Tris-HCl, pH 8.45. The cathode running buffer consisted of 0.1 mol/L Tris-HCl, pH 8.25, 0.1 mol/L tricine, and 0.1% SDS, and the anode buffer consisted of 0.2 mol/L Tris-HCl, pH 8.9. Samples were diluted 2-fold with 2% SDS, 5 mmol/L Tris-HCl, pH 6.5, 20% sucrose, 0.05% bromophenol blue, and 100 μmol/L β-mercaptoethanol (β-ME), with the addition of 6 mol/L urea for T-PAGE. Prepared samples were boiled for 5 minutes and loaded onto the gel. Gels were stained with Coomassie blue followed by silver23 or transferred to a nitrocellulose (for Western blotting) or polyvinylidine difluoride (for amino acid microsequencing) membrane using a wet transfer apparatus (Bio-Rad) with 10 mmol/L 3-cyclohexylamino-1-propanesulfonic acid (CAPS), pH 11.0, for 16 hours at 4°C. Western blot analysis was carried out as described previously.1 The primary antibodies (Figure 1) were detected with horseradish peroxidase conjugated to goat anti-mouse IgG reagent (NEN-Mandel). Epitope mapping of the MAbs 8I-7 and 8I-7b (MAb TnT overexposed/overloaded, Figure 2A), and 8I-7c (Figure 2B) to 20 μg (Figure 2B) or 40 μg (MAb TnT overexposed/overloaded, Figure 2A), and overexposure of blots was used to facilitate visualization of weak associations between the MAbs and the TnI products.

Alkaline urea PAGE was performed as outlined by Head and Perry.24 The amount of protein loaded onto the gel was increased from 5 μg (Figure 2B) to 20 μg (Figure 2A) or 40 μg (MAb TnT overexposed/overloaded, Figure 2A), and overexposure of blots was used to facilitate visualization of weak associations between the MAbs and the TnI products.

Two-dimensional (2D) electrophoresis of tissue homogenates was adapted from a standard protocol (Bulletin 1144) from Bio-Rad, except that we used T-PAGE for the second dimension. Proteins were resolved in the first dimension by isoelectric focusing on a mini-Protein II isoelectric focusing gel electrophoresis apparatus (Bio-Rad) using an ampholyte mixture of 90% pH 3.5 to 10.0 (Sigma) and 10% pH 4.0 to 6.5 (Pharmacia). Protein resolution in the second dimension was carried out by T-PAGE with 6 mol/L urea. The 2D gels were stained with Coomassie blue or transferred to nitrocellulose for Western blot analysis.

Mass Spectrometry

Approximately 50 to 200 μg of each lyophilized RP-HPLC peak obtained from both affinity columns was analyzed by matrix-assisted laser desorption/ionization (Kratos) or electrospray (Fisons VG Quattro) mass spectrometry. From these masses, the possible TnT, TnC, and TnT fragments were determined using the SwissProt protein database and the PeptideMass tool from the WorldWide Web molecular biology server of the Swiss Institute of Bioinformatics (http://expasy.hcuge.ch/). Protein sequences from rcTnI, mouse cTnC (mcTnC), and cTnI were sequentially clipped from the amino and carboxyl termini until a match to the observed monoisotopic mass was found. Masses and amino acid sequences of the TnC and TnT components of the high molecular weight complexes were determined by considering all amino acid sequences that conformed to the necessary restrictions, as described in Results.

Results

Time Dependence of TnI Modification

In this study we have identified the TnI degradation products and characterized additional modifications within the tropinin complex that are induced by varying degrees of ischemia/reperfusion injury. Figure 2A shows Western blots of T-PAGE–separated (with 6 mol/L urea) left ventricular tissue from isolated Langendorff-perfused rat hearts that had undergone 0, 15, or 60 minutes of no-flow ischemia followed by 45 minutes of reperfusion. With this gel system, the visualization of 6- to 70-kDa proteins is facilitated, but some proteins, including the troponin subunits, do not migrate according to their molecular weights. Initial Western blot analysis of modified TnI products was performed using the following series of MAbs that recognize various defined regions of TnI: AM-IN (N-terminal), 8I-7 (central), and 3I-35 (C-terminal) (Figure 1). Control hearts (0/45) showed little degradation of TnI (5.6%) (Figure 2A, Table 1A). A substantial increase in the quantity of degradation was shown under ischemia/reperfusion, with increases in the amount and type of TnI products corresponding to increases in the severity of the ischemic insult. The mildly ischemic condition of 15/45, in which few or no proteins are released indicating no cellular necrosis,1 resulted in modification of 48% of total TnI, because of production of a 22-kDa TnI degradation product and 3 high molecular mass complexes (~55, 66, and 75 kDa; combined, 16.9% of total TnI) (Figure 2A, Table 1A). Analysis of 12.5% SDS-PAGE demonstrated that these high molecular mass complexes are all in fact between 30 and 40 kDa (Figure 2B). The proportion of the 22-kDa product to total TnI content did not change substantially in tissue from
This is in spite of an increase in the quantity of TnI degradation (64.7%, Table 1) due to changes in the type of TnI modification. Severe ischemia/reperfusion injury resulted in loss of the high molecular weight complexes, with further degradation of TnI to 2 products with molecular masses of 16 and 15 kDa (15.1% and 17.2% of total TnI, respectively; Table 1), as well as degradation of TnT (Figure 2A). The quantification of MAb 8I-7 Western blots was confirmed with anti-TnI MAb E2 (epitope rcTnI residues 136–147), with all modified TnI products within a 5% error of those obtained with MAb 8I-7 (data not shown). We recognize, however, that the inherent assumption of equal binding affinity of these MAbs for all products does not necessarily hold. Significant cellular necrosis occurs with 60/45,1 and analysis of 60/45 reperfusion effluent demonstrated preferential release of the 22-kDa TnI degradation product from necrotic cardiomyocytes following severe ischemia, along with TnI-containing covalent complexes. C, 2D electrophoresis of left ventricular tissue samples from 60/45 tissue, separated in the first dimension by a pH gradient produced by an ampholyte mixture of 90% pH 3.0 to 10.0 and 10% pH 4.0 to 6.5 and in the second dimension by T-PAGE with 6 mol/L urea. The gels were stained with Coomassie blue or transferred to nitrocellulose for Western blotting with MAb 8I-7. Phosphate groups carry a negative charge, so phosphorylation of TnI would produce a spot(s) at the same molecular weight but at a lower pH. Intact TnI occurs as a series of points, indicating that some TnI is phosphorylated. The 22-kDa TnI degradation product occurs primarily as a single point, with a faint point occurring at lower pH, indicating that the majority of this product is not phosphorylated. Other TnI degradation products also occur as a single point. All protocols were performed as described in Materials and Methods.
TABLE 1. Progressive Alteration of TnI With Increasing Severity of Ischemia

<table>
<thead>
<tr>
<th>Ischemia/Reperfusion-Induced TnI Product</th>
<th>A. Left Ventricle Tissue Percentage of Total TnI*</th>
<th>B. Anti-TnI MAb Affinity Chromatography Percentage of Total TnI‡</th>
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<tr>
<td></td>
<td>0/45†</td>
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<tr>
<td>Covalent complexes§</td>
<td>0</td>
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<tr>
<td>rcTnI</td>
<td>94.4%</td>
<td>52.2%</td>
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<tr>
<td>rcTnI degradation products</td>
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<td></td>
</tr>
<tr>
<td>~22 kDa</td>
<td>5.6%</td>
<td>24.1%</td>
</tr>
<tr>
<td>~16 kDa</td>
<td>0</td>
<td>0%‡</td>
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<tr>
<td>~15 kDa</td>
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</table>

*The ischemia/reperfusion-induced modified TnI products observed in T-PAGE-separated left ventricular tissue. Tissues were obtained from isolated perfused rat hearts (n=3) that underwent 0/45, 15/45, or 60/45. The quantity of each TnI component was determined as a percentage of the total TnI (intact and modified) present in each tissue sample, quantified from 8I-7 MAb Western blots (Figure 2A). Only the modified TnI products positively identified in Table 2 are included here, identified by their apparent molecular mass on T-PAGE (Figure 2A).†Control tissue, from isolated rat hearts that experienced no ischemic episode, but 45 minutes of reperfusion.‡The ischemia/reperfusion-induced modified TnI products observed from 8I-7 MAb affinity chromatography of TnI (Figure 4). The quantity of each TnI component was determined as a percentage of the total in each sample.§Quantity of the 2 TnI-containing covalent complexes combined.¶ND indicates not determined, because quantities <2% of total TnI could not be accurately determined.¶A low molecular mass product (≈18 kDa) was seen with 15/45, but could not be identified, and is not included in the table. It was, however, included in the calculation of percentage total TnI.

Western blot analysis of 2D electrophoresis of 60/45 tissue homogenate revealed at least 2 spots, or phosphorylation states, for intact TnI, with only 1 spot for each of the degradation products (Figure 2C). Since phosphorylation adds negative charge to proteins, phosphoproteins appear as spots at the same molecular weight as when unphosphorylated, but at lower pH. The degradation products are thus not phosphorylated.

**Isolation and Identification of Modified TnI Products**

Affinity chromatography was used to isolate the various modified TnI products that were observed by Western blotting of the ischemia/reperfused tissue (Figure 2). TnC affinity chromatography of the 60/45 tissue sample resulted in the elution of a single TnI product by RP-HPLC (Figure 3A). This product was collected and analyzed by mass spectrometry, which detected a mass of 22 144±7.8 Da (Figure 3B). The identity of this product as the 22-kDa TnI degradation product was confirmed by Western blotting (data not shown). Analysis of the rcTnI amino acid sequence identified a single sequence, rcTnI residues 1–193 (mass 22 152 Da), which was the only sequence that corresponded to the observed mass. The identification of TnI 1–193, a C-terminal degradation product of rcTnI, confirmed the weak interaction with the C-terminal MAb 31-35 (Figure 2A) and the previously reported blocked N-terminus as revealed by unsuccessful amino acid microsequencing.¹

Although TnI 1–193 is the primary TnI degradation product observed with 15/45, more severe ischemia results in further degradation of TnI (Figure 2A). These other products were isolated by anti-TnI MAb 8I-7 affinity chromatography of 15/45 and 60/45 left ventricle tissue. Several protein products were isolated from fractions that bound to the MAb...
81-7 affinity column by RP-HPLC (Figure 4A). While 15/45 tissue did not yield sufficient protein for further analysis, 60/45 tissue produced 4 distinct peak groups (referred to as peaks 1 through 4, Figure 4A) that were further analyzed by mass spectrometry (Table 2), T-PAGE with 6 mol/L urea (Figure 4B), Western blotting (Figure 4C), and alkaline urea PAGE (Figure 4D).

Peak 1 comprised a series of degradation products (molecular masses 25, 27, and 33 kDa, Figure 4B), and Western blotting (Figure 4C) confirmed their identity as the TnT degradation products observed previously (Figure 2A). Peak 2 comprised a high molecular weight covalent complex, in addition to several smaller degradation products. The covalent complex was shown to comprise TnI (MAb 8I-7 overexposure, Figure 4C) and TnC (alkaline urea PAGE, Figure 4D). The other components of peak 2 were shown to be intact rcTnI, the 22-kDa product (TnI 1–193), and the 16- and 15-kDa products (MAb 8I-7 overexposure, Figure 4C). Mass spectrometry of this peak returned masses of 32,734 ± 9 Da, 15,348 ± 15 Da, and 14,096 Da (Table 2). Analysis of the rcTnI amino acid sequence identified single

<table>
<thead>
<tr>
<th>Ischemia/Reperfusion- Induced TnI Product*</th>
<th>Source†</th>
<th>Immunoreactivity With MAb‡</th>
<th>Alkaline Urea PAGE§</th>
<th>Putative Identification¶</th>
<th>Observed Mass, Da ± SE</th>
<th>Theoretical Mass, Da¶††</th>
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<tr>
<td>Covalent complexes</td>
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<td></td>
<td></td>
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<tr>
<td>~66 kDa</td>
<td>Peak 3</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>−</td>
<td>rcTnI(1–193)/ TnT(191–298)</td>
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<tr>
<td>~55 kDa</td>
<td>Peak 2</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>−</td>
<td>rcTnI(1–193)/ TnC(1–94)</td>
</tr>
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<td>rcTnI degradation products</td>
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<td>~22 kDa</td>
<td>Peak 2</td>
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<td>±</td>
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<td>rcTnI 63–193</td>
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<td>±</td>
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<td>rcTnI 73–193</td>
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<td>~15 kDa</td>
<td>Peak 2</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>rcTnI 35–193</td>
</tr>
</tbody>
</table>

* TnI products identified by their apparent molecular masses from T-PAGE with 6 mol/L urea (Figure 2A).
† The source of the TnI products indicates the peak from RP-HPLC analyzed 8I-7 affinity column fractions of 60/45 tissue (Figure 4).
‡ Immunological analysis of protein products to MAb (Figure 1) from Western blotting (Figures 2A and 4C), given as strong (††), weak (††), and no binding (−).
§ Electrophoretic mobility in alkaline urea PAGE (Figure 4), given as mobile (+, ie, containing TnC) or nonmobile (−, not containing TnC).
¶ The amino acid sequence(s) of proteins that are the theoretical best match to the observed masses. Intact rcTnI, mcTnC, and rcTnT are 210, 161, and 298 amino acids long, respectively.
# Mass determined by electrospray mass spectrometry.
** Mass determined by matrix-assisted laser desorption/ionization mass spectrometry.
†† The difference between the observed and theoretical masses is equal to that of a sodium ion (molecular mass, 35 Da), which is commonly found associated with mass spectrometrically analyzed proteins (as a result of the ionization process).

Figure 3. Isolation and identification of the 22-kDa TnI degradation product by TnC affinity chromatography. A, RP-HPLC elution profile of proteins isolated by TnC affinity chromatography of left ventricle tissue homogenates from rat hearts that experienced 60/45. The column was washed with 20 mmol/L Tris-HCl, pH 7.4, 50 mmol/L potassium chloride, and 1 mmol/L calcium chloride, and then bound proteins were eluted with 65 mmol/L glycine-HCl, pH 3.2. B, Electrospray mass spectrometry analysis of the RP-HPLC peak eluted at 39% acetonitrile (CH₃CN; shown in panel A). Analysis of the rcTnI amino acid sequence identified single
sequences corresponding to the 2 smaller masses: rcTnI 63–193 (mass 15 377 Da) and rcTnI 73–193 (mass 14 096 Da). Peak 3 comprised a large number of high molecular weight products. Western blots demonstrated the presence of 1 TnI-containing covalent complex and intact rcTnI, with little further degradation (MAb 8I-7 overexposure, Figure 4C). The covalent complex in peak 3 did not contain TnC (Figure 4D) but did contain TnT (MAb TnT overexposed/overloaded, Figure 4C). Mass spectrometry of peak 3 returned a mass of 32 872 \pm 9 Da for the complex. Peak 4 comprised a covalent complex and a protein with electrophoretic migration and staining properties similar to those of cTnC (Figure 4B). TnT was shown to be a component of the covalent complex (MAb TnT overexposed/overloaded, Figure 4C), while alkaline urea PAGE confirmed that both of these products contained TnC. Importantly, TnI was not present in this covalent complex (MAb 8I-7, Figure 4C and 4D). Mass spectrometry of peak 4 returned a mass of 33 595 \pm 32 Da for the complex and a mass of 18 420 Da for the smaller product. The 18 420-Da product is similar in mass to mcTnC, but since the sequence for rcTnC is not known, further analysis was precluded. This analysis is summarized and correlated to the Western blotting data in Table 2.
The cumulative information about the covalent complexes from Western blotting, mass spectrometry, and alkaline urea PAGE, as summarized in Table 2, was used to assign identities to the 2 TnI-containing covalent complexes. There was insufficient information about the 33 595-Da TnT-TnC covalent complex to perform similar analyses for it, and so it is not included in Table 2. The 2 TnI-containing complexes have identical immunoreactivities to that of the TnI MAbs (Figures 2A and 4C), which correspond to that of TnI 1–193 (Figure 2A), not intact TnI (Figure 2A). Few processes are known to cause covalent cross-linking, except through the enzyme TGase, which is known to target the troponins.26 TGase is a ubiquitous enzyme that forms isopeptide bonds between specific glutamine and lysine residues,26,27 and the C-terminal residue of rcTnI 1–193 is a lysine. Amino acid microsequencing of these complexes was unsuccessful, likely because of a blocked N-terminus. Even so, the amino acid sequences of mcTnC and rcTnT were analyzed to determine whether there were sequences that would sum with rcTnI 1–193 to the appropriate mass and have either a blocked N-terminus or an N-terminal glutamine residue. While isopeptide bonds are still susceptible to hydrolysis, the sequence produced by linking TnI lysine 193 to the N-terminus of another protein or degradation product would be linear and not susceptible to Edman degradation. This led to the identification of single sequences, mcTnC 1–94 (theoretical mass of complex with TnI 1–193 of 32 730 Da) and rcTnT 191–298 (theoretical mass of complex with TnI 1–193 of 32 871 Da), with these properties (Table 2). The N-terminus of rcTnC is known to be blocked, and rcTnT residue 191 is indeed a glutamine.

**Discussion**

**TnI Degradation Is a Progressive and Selective Process**

Several groups have examined the cellular modifications associated with myocardial ischemia/reperfusion injury, including the degradation of myofilament proteins such as TnI and α-actinin. Westfall and Solaro4 first postulated degradation of TnI and TnT following 60 minutes of complete global ischemia in rat hearts. Gao et al2 detected the presence of a TnI degradation product in mildly ischemic (20-minute ischemia and 20-minute reperfusion) isolated perfused rat hearts, and this injury was prevented by low calcium/low pH perfusion. Hein et al6 demonstrated changes in the localization pattern of myosin, actin, tropomyosin, and TnT as early as 10 minutes after the onset of ischemia in cardiomyopathic human left ventricular tissue. Recently, our laboratory demonstrated that TnI and α-actinin in isolated rat hearts are specifically degraded during 15/45, 60 minutes of ischemia with no reperfusion, and 60/45.1 Here we have identified and characterized the modifications to TnI under mild and severe ischemia/reperfusion and demonstrated a progressive and selective pattern to the modification process, whereby increases in the severity of ischemia result in increases in the extent of TnI degradation.

The mild ischemia/reperfusion condition of 15/45 results in the activation of 2 processes through increases in intracellular calcium,2 which are protease activity and covalent cross-linking activity. There is substantial indirect evidence suggesting that the calcium-dependent cysteine protease calpain is responsible for the production of the 22-kDa TnI C-terminal degradation product, identified here as rcTnI 1–193 (Figure 5, Table 2). Calpain may also be implicated in the N-terminal proteolytic activity, which produces further degradation of TnI to residues 63–193 and 73–193, with more severe ischemia/reperfusion (60/45). Calpain is known to cleave both rcTnI and rcTnT in vitro,18 and treatment of skinned trabecula fibers with calpain produces a dysfunction and TnI degradation product similar to those seen in ischemia/reperfused fibers.3 To date, there is no known consensus...
sequence specifically targeted by calpain, and the 3 rcTnI cleavage sites identified here share no significant sequence similarity. Currently, there is a dispute over whether sequences rich in proline, glutamic acid, serine, and tyrosine residues (“PEST” sequences) are involved in recruitment of calpain to cleavage sites.28–30 The processes by which the activated enzyme is localized to the thin filament are only now being postulated.

The functional implications of TnI degradation are more readily interpreted now that the amino acid sequence of the degradation products is known. Studies of rcTnI deletion mutants comprising amino acid residues 1–199, 1–188, and 1–151 have shown that the 1–188 and 1–151 mutants have impaired abilities to bind TnC and inhibit actin-activated myosin ATP hydrolysis compared with both the 1–199 mutant and intact rcTnI.31 Neighboring residues between rcTnI 150–180 have been implicated in both TnC and actin-tropomyosin binding.32 The decrease in calcium sensitivity or in maximum force seen in triton-skinned trabeculae fibers from ischemia/reperfused rat hearts cannot yet be attributed to rcTnI 1–193, the primary ischemia-induced degradation product.1,7 However, substitution of the troponin complex from stunned myocardium into control skeletal muscle fibers demonstrated a decrease in the calcium sensitivity of force generation with respect to controls.33 Modification to the troponin complex may be directly responsible for the myofibrillar dysfunction observed with ischemia. While substantial intact TnI exists within the ischemic cells (Table 1A), several groups have found that even low levels of modified contractile proteins can significantly alter contractility.34,35 To further explore the functional implications of TnI degradation, transgenic mice have been produced that express mcTnI 1–193 in the myocardium (Dr A. Murphy, Johns Hopkins University, personal communication, 1998). These mice demonstrate depressed left ventricular function.

In contrast, the functional characteristics of the rcTnI N-terminus are well defined, with essential roles in TnC binding and the cooperativity of actin-tropomyosin binding.10,32 The progressive loss of this region with severe ischemia may result in a reduction in the ability of calcium to regulate the contraction of cardiac muscle.

Previous work has shown that 15/45 conditions do not result in cellular necrosis over control levels, while 60/45 conditions do result in necrotic release of cellular proteins.1 These experimental conditions approximate the clinical phenomena of myocardial stunning and acute myocardial infarction. The rcTnI component of these proteins was shown here to be primarily rcTnI 1–193 and not intact rcTnI. Also present in the reperfusion effluent were TnI-containing covalent complexes. It has been established that cTnI products are found in the blood after an ischemic episode, and serum TnI levels are now used as a clinical diagnostic for acute myocardial infarction.36 Recent work by Wu et al37 has demonstrated the presence of a binary complex of cTnI-cTnC, a ternary complex of cTnI-TnC-cTnC, free cTnT, and no free cTnI in serum from patients who had experienced acute myocardial infarction. Our results, along with those of Wu et al37 and others (see references in Wu et al37), suggest that current cTnI serum diagnostics may predominantly detect modified TnI products (ie, TnI 1–193 and covalent complexes) and little if any intact protein.

While modified TnI products were produced with both mild and severe ischemia/reperfusion, a population of TnI was protected from degradation (Figure 4C, compare peaks 2 and 3, MAb 81-7). Quantification and comparison of peaks 2 and 3 obtained from RP-HPLC analysis of bound fractions from 81-7 affinity chromatography of 60/45 tissue demonstrated that while 29% of TnI was degraded in peak 2, only 9% was degraded in peak 3 (Table 1B). In vitro studies have shown that phosphorylation of cTnI by PKA reduces the sensitivity of cTnI to degradation by calpain.18 Two-dimensional electrophoresis is a powerful tool for elucidating phosphorylation states and determined that the TnI degradation products were indeed unphosphorylated, despite significant levels of phosphorylated intact TnI (Figure 2C). However, the 15- and 16-kDa TnI products have lost the N-terminal phosphorylation sites at serine residues 23 and 24. Recent work has shown that phosphorylation of TnI and TnT is increased following myocardial infarction in dogs,38 confirming that the amount of phosphorylated TnI seen in the 2D gel is not excessive. Interestingly, the calpain-mediated proteolysis of other proteins, in particular tau, is inhibited by substrate phosphorylation.39,40 In the case of connexin-32, phosphorylation prevents calpain-mediated proteolysis but not degradation by papain, α-chymotrypsin, proteinase K, or trypsin.41 Phosphorylation of TnI by PKA produces a change in the fluorescence properties of tryptophan 192 of cTnI, indicating that the conformation of the C-terminal region of cTnI is altered.42 In our laboratory, we have found that treatment of experimental animals with isoproterenol, which activates PKA-dependent pathways, provides protection against TnI degradation (Taylor and Van Eyk, unpublished data, 1998). The “protected” population of TnI observed in Figure 4C may represent phosphorylated rcTnI.

Covalent Complexes Are Formed With Mild Ischemia/Reperfusion

The identification of binary covalent complexes formed under mild ischemia/reperfusion injury, comprising TnI 1–193 and, respectively, TnC 1–94 and TnT 191–298, suggests the activity of a cardiac tissue TGase isofrom. TGases are activated by calcium to catalyze the formation of isopeptide bonds between specific glutamine residues and primary amines, preferentially the amine group of adjacent lysine residues.43 Nakaoaka et al44 demonstrated that α-adrenergic receptors of the heart activate a phospholipase C enzyme through a GTP binding protein, Gα, which they further demonstrated to exhibit TGase activity. It has since been shown that all tissue TGases contain GTP binding domains,27,45,46 and the TGase activity of Gα is inhibited by the binding of GTP.27,45,46 Furthermore, Gα expression is down-regulated in the failing human heart, despite an increase in α-adrenergic receptor expression; however, there was no correlation between TGase activity and Gα protein levels.47 Hwang et al47 suggest that Gα activation may be regulated by an unknown inhibitory factor. However, Gα may not be the only TGase in cardiomyocytes, and so the exact ischemia/
reperfusion-induced mechanism of TGase activation remains unclear.

The TnI/TnT complex may be the result of a covalent bond between TnI lysine 193 and TnT glutamine 191, while the TnI/TnC complex may be the result of a covalent bond between TnI lysine 193 and an internal glutamine residue in the TnC 1–94 sequence (Figure 5). Despite the absence of a sequence for rcTnC, there is a high degree of homology within cTnC isoforms at both the amino acid and structural levels between mammalian species. The low-affinity regulatory calcium binding site of the TnC N-terminus is known to interact with the C-terminal region of cTnI, such that TnI lysine 193 is likely linked to an N-terminal glutamine on TnC 1–94. The C-terminal region of cTnC (residues 126–136), which is absent in the ischemia-induced TnI/TnC complex, is believed to contain an anchoring region for rcTnI that facilitates the movement of the inhibitory region of rcTnI for cTnC. The covalent complex regulates the calcium binding site of TnC, anchors rcTnI to cTnC, and may modulate the affinity of the inhibitory region of rcTnI for cTnC.

The covalent complexes observed in myocardium with mild ischemia/reperfusion were greatly reduced with more severe conditions, implying that the TGase-catalyzed isopeptide bonds may have been broken. Although a portion of the covalent complexes were released into the 60/45 reperfusion effluent (Figure 2A and 2B, Table 1A), this cannot account for all of the complexes observed in 15/45 tissue (Figure 2A). Since the isopeptide bond is not susceptible to protease activity, the most probable mechanism whereby these links could be broken is through isopeptidase activity, whereby the isopeptide bond is hydrolyzed. While the presence of a tissue isopeptidase is currently under dispute, TGases themselves show significant internal isopeptidase activity at high concentrations. It is possible that escalating TGase activation following longer ischemic episodes results in a reversal of the covalent linkages formed under milder conditions. The progress of TGase-induced ischemia/reperfusion injury may therefore be self-limiting.

The involvement of TGases in ischemia/reperfusion injury has been suggested previously by Gorza et al., who observed unidentified covalent complexes containing TnI in ischemia/reperfused cardiomyocytes accompanied by changes in TnT immunoreactivity in cryosections of ischemic rat hearts with 60 minutes of ischemia followed by 30 minutes of reperfusion. However, Gao et al. did not see covalent complexes with 20 minutes of ischemia followed by 20 minutes of reperfusion. This may reflect the affinity of the MAbs used in analysis of the degradation products and different periods of reperfusion. The lack of a clear activating signal for TGase activity prevents any further conclusions. However, the significant TnT degradation observed here with severe ischemia/reperfusion supports the modulation of TnT immunoreactivity observed by Gorza et al. and the production of the covalently complexed TnT 191–298. This degradation results in loss of the majority of the rcTnI binding region on cTnT (residues 152–209) and loss of N-terminal tropomyosin binding sites. The formation of a covalent complex may help to preserve the association of the troponins.

Possible Interactions Between Calpain and TGase

The relationship between proteolytic and covalently linking processes may reflect the balance of the regulatory processes that ultimately govern cell death. The release of cellular proteins into the reperfusion effluent with severe ischemia implies significant necrosis, the acute process whereby the cell membrane of severely damaged cells ruptures. However, DNA fragmentation studies have demonstrated that a portion of cardiomyocytes are destroyed through the highly regulated process of apoptosis, whereby specific stimuli result in the transcription of cell death genes, and the cell is dismantled into discrete “packages” (ie, proteins are not released). Hypoxic damage in the rat liver is prevented by in vivo transfection with Bcl-2, a known apoptosis inhibitor. Bcl-2 treatment also prevents potassium cyanide–induced necrotic death in PC 12 cells, suggesting a link between apoptosis and necrosis. Tissue TGases have been implicated in the cellular remodeling associated with apoptosis, and TnT cross-linking has been found in human apoptotic cardiomyocytes. Calpain is known to be activated under both apoptotic and nonapoptotic conditions. Interactions between TGases and calpain have been previously suggested, since factor XIII (a blood clotting factor and a TGase) is proteolytically activated. However, the tissue-type TGases lack the N-terminal extension that is cleaved from factor XIII. Even so, tissue TGases have been shown to be in situ substrates for calpain degradation, which is inhibited in the presence of GTP but inactivates the enzyme. The pathological interactions between TGases and calpain are not yet known. Since the loss of covalent complexes with severe ischemia is associated with the further degradation of TnI and TnT, these covalent attachments may provide some protection against further degradation. This possible protective function indicates that the 2 processes are associated but not complementary (ie, destruction versus preservation). The balance between these 2 processes within the cell may determine whether necrosis or apoptosis is achieved with increasing muscle damage.

Proposed Model for Ischemia/Reperfusion-Induced TnI Modification

The onset of ischemia/reperfusion injury involves an increase in cellular calcium concentration. It is this increase in cellular calcium that may result in the activation of both proteolytic and TGase activities, possibly through interactions between calpain and a tissue TGase. The selective degradation of troponin (to TnI 1–193, cTnC to TnC 1–94, and cTnT to 191–298) may occur either before or after the formation of covalent links between these components. The balance between the formation of the stabilized covalent complex and the amount of troponin degradation may determine whether necrotic or apoptotic pathways will be activated. While stunning conditions have not been shown to produce apoptotic death, pathways that may eventually lead to apoptosis, as well as those which may lead to necrosis, may be activated. As a result, TGase and calpain activities are modulated such that covalent complexes are maintained, broken, or released into the reperfusion effluent, depending on the severity of the ischemic insult. The factors that
modulate the activity of these enzymes may include pH, intracellular calcium, and ATP levels, as well as many others. With severe ischemia/reperfusion, TnI 1–193 released from broken covalent complexes is further selectively degraded from the N-terminus, to TnI 63–193 and TnI 73–193, producing a progression of degradation products. As cellular damage proliferates, necrosis ensues. In cells with only moderate TGase activation, or moderate protease activation, under more severe ischemia/reperfusion, necrosis averted through the stabilizing actions of TGase cross-links. This population of cells is given the opportunity to gain the possible advantages of undergoing apoptosis instead of necrosis. This model thus proposes that there are 2 populations of cardiomyocytes, necrotic and apoptotic, which may represent histological and/or functional differences. The spectrum of damage caused by myocardial ischemia/reperfusion injury is the physiological manifestation of the protein modifications induced by the ischemia/reperfusion insult and the balance between necrosis and programmed cell death processes. The result is the selective and progressive degradation of cTnl, which is accompanied by the formation of covalent complexes between the troponin subunits.

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Ischemia-Induced Modification of TnI


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