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

Jason L. McDonough, D. Kent Arrell, Jennifer E. Van Eyk

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\(^2,3,17\); 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 likely responsible for the degradation of TnI.\(^2,18\) We have also 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 \(-70^\circ\text{C}\) 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 \(\mu\)mol/L leupeptin, 2.1 \(\mu\)mol/L pepstatin A, and 50 \(\mu\)mol/L phenylmethylsulfonylfluoride) on ice. Aliquots of tissue homogenates for electrophoresis were stored at \(-20^\circ\text{C}\) until use. Remaining tissue homogenates for affinity chromatography were dialyzed at 4\(^\circ\text{C}\) against 2 mol/L urea, 1 mol/L potassium chloride, 1 mmol/L DTT, and 20 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\(^\circ\text{C}\), and the supernatant was used for further analysis. Total protein concentration of tissue homogenate supernatants and reperfusion effluents were determined according to Lowry et al.\(^20\) Bovine cTnI, cTnC, and cTnT and rabbit sTnC were isolated and purified as described 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 cyanoanide 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 0.86 mol/L MOPS, pH 8.0, to neutralize the pH to 7.5. After lyophilization, fractions were resuspended in 0.05% 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 Specialists 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% aqueous 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 mmol/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 polyvinylidene 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 goat anti-mouse IgG-conjugated red fluorescence (red fluorescence dye; Molecular Probes, Eugene, Oregon) and secondary antibody (MAb) 8I-7 (Spectral Diagnostics, Figure 1) was cross-linked to cyanoanide 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 0.86 mol/L MOPS, pH 8.0, to neutralize the pH to 7.5. After lyophilization, fractions were resuspended in 0.05% aqueous tridifluoroacetic 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 Specialists 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% aqueous 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).

**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 TnI, 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 TnC (mcTnC), and rcTnT 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 tropo

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1. The MAb AM-IN was obtained from, and the epitope mapped by, Dr J. Ladenson (Washington University, St. Louis, Mo) and binds the N-terminal region of TnI. MAb TnT clone JLT-12 was obtained from, and the epitope mapped by, Dr J. Ladenson (Washington University, St. Louis, Mo) and binds the N-terminal region of TnI. MAb 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 TnC (mcTnC), and rcTnT 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 tropo
60/45 hearts (21.3%, Table 1). 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 the cell with respect to intact TnI (Figure 2B). Previously published analysis has demonstrated that no proteins are released into the reperfusion effluent with 15/45, and so no data are included.1 Analysis of 60/45 reperfusion effluent with MAb 3I-59, which preferentially binds to the covalent complexes, demonstrated that TnI-containing covalent complexes are also found in the effluent (Figure 2B). Previously we used a different anti-TnI antibody (MAb E2) which failed to demonstrate the presence of TnI products in the reperfusion effluent.1 This is similar to the results shown...
TABLE 1. Progressive Alteration of TnI With Increasing Severity of Ischemia

<table>
<thead>
<tr>
<th>Ischemia/Reperfusion-Induced</th>
<th>A. Left Ventricle Tissue Percentage of Total TnI*</th>
<th>B. Anti-Tnl MAb Affinity Chromatography Percentage of Total Tn†</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnI Product</td>
<td>0/45†  15/45  60/45</td>
<td>Peak 1  Peak 2  Peak 3  Peak 4</td>
</tr>
<tr>
<td>Covalent complexes§</td>
<td>0      16.9%  3.1%</td>
<td>0      ND†   ND   0</td>
</tr>
<tr>
<td>rcTnl</td>
<td>94.4%  52.2%  35.3%</td>
<td>0      71.7%  91.4%  0</td>
</tr>
<tr>
<td>rcTnI degradation products</td>
<td>0      0%     0%</td>
<td>0      0%      0%</td>
</tr>
<tr>
<td>~22 kDa</td>
<td>5.6%    24.1%  21.3%</td>
<td>0      25.5%  6.4%  0</td>
</tr>
<tr>
<td>~16 kDa</td>
<td>0      0%     0%</td>
<td>0      2.8%   2.2%  0</td>
</tr>
<tr>
<td>~15 kDa</td>
<td>0      0%     0%</td>
<td>0      ND†   ND   0</td>
</tr>
</tbody>
</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 60/45 left ventricular tissue (Figure 4) were quantified from 8I-7 MAb Western blots (see 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.

The ability of the anti-TnI MAbs to recognize different epitopes on TnI was exploited in the initial characterization of the modified TnI products observed in ischemia/reperfused myocardium. We have previously shown that the 22-kDa TnI degradation product is a product of C-terminal proteolysis because of its weak interaction with MAb 10F2 (epitope rcTnI amino acid residues 188–199). This is confirmed here by its weak associations with MAb 3I-35 and AM-IN MAbs but only weakly with 8I-7 and AM-IN clearly detect the 22-kDa degradation product. MAbs E2 and 3I-35 only weakly associate with the 22-kDa degradation product. MAbs E2 and 3I-35, indicating C-terminal degradation, possibly similar to that of the 22-kDa degradation product (compare response to 8I-7 versus 3I-7, Figure 2A). The 66- and 75-kDa covalent complexes interacted strongly with the anti-TnT MAb. The 3 complexes may be degradation products of TnI/TnC (55 kDa), TnI/TnT (66 kDa), and TnT/TnC (75 kDa) (Table 2).

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 3I-35 (Figure 2A) and the previously reported blocked N-terminus as revealed by unsuccessful amino acid microsequencing.

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 Da, 15 348 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>
</tr>
</thead>
<tbody>
<tr>
<td>Covalent complexes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>~66 kDa Peak 3</td>
<td></td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>–</td>
<td>rcTnI(1–193)/TnT(191–298)</td>
</tr>
<tr>
<td>~55 kDa Peak 2</td>
<td></td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>–</td>
<td>rcTnI(1–193)/TnC(1–94)</td>
</tr>
<tr>
<td>rcTnI degradation products</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~22 kDa Peak 2</td>
<td></td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>–</td>
<td>rcTnI 1–193</td>
</tr>
<tr>
<td>~16 kDa Peak 2</td>
<td></td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>–</td>
<td>rcTnI 63–193</td>
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<tr>
<td>~15 kDa Peak 2</td>
<td></td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>rcTnI 73–193</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 (1, ie, containing TnC) or nonmobile (2, 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).**

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).

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**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 a single sequence of appropriate mass, rcTnI residues 1–193, with mass 22 152 Da. All protocols were performed as described in Materials and Methods.
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 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 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.

Figure 4. Isolation of modified TnI products by anti-TnI antibody affinity chromatography. A, RP-HPLC elution profiles of proteins that bound to the 8I-7 MAb affinity column from left ventricle tissue homogenates from rat hearts that experienced either 15/45 or 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 bound fractions were eluted with 65 mmol/L glycine-HCl, pH 3.2. Arrows indicate elution times of purified intact cTnI, cTnC, and cTnT. B, T-PAGE (with 6 mol/L urea) separation of RP-HPLC fractions obtained from 8I-7 MAb affinity chromatography of 60/45 tissue and purified cTnC. Peaks (labeled 1 to 4, panel A) were collected from RP-HPLC, separated by urea T-PAGE, stained with Coomassie blue and silver (B), or transferred to nitrocellulose. C, Western blot analysis of the T-PAGE-separated RP-HPLC peaks 1 to 4 with the MAb 8I-7 and TnT. Overloading of samples and/or overexposure of Western blots was necessary to visualize the covalent complexes because of their low amounts. D, Alkaline urea PAGE analysis of peaks 2 to 4 (since peak 1 contains TnT). TnI and TnT or their fragments will only migrate into the gel if complexed to TnC. Detailed procedures are described in Materials and Methods.
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. TGase is a ubiquitous enzyme that forms isopeptide bonds between specific glutamine and lysine residues, 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 Solaro first postulated degradation of TnI and TnT following 60 minutes of complete global ischemia in rat hearts. Gao et al 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 al 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. 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, 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, and treatment of skinned trabecula fibers with calpain produces a dysfunction and TnI degradation product similar to those seen in ischemia/reperfused fibers. 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 contractile function.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.36,37 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. 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.

Recent work by Wu et al17 has demonstrated the presence of a binary complex of cTnC-cTnI, a ternary complex of cTnT-cTnI-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 al17 and others (see references in Wu et al17), 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, MA8 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 substbrane 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 isoform. 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 Nakaoka 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 downregulated 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/
Ischemia-Induced Modification of TnI

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. 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 processes are associated but not complementary (ie, destruction versus preservation). The balance between these 2 processes within the cell may therefore be self-limiting.

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 physiopathological 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 cTnI, which is accompanied by the formation of covalent complexes between the troponin subunits.

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