Alterations in Myofibrillar Function and Protein Profiles After Complete Global Ischemia in Rat Hearts

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We studied changes in myofibrillar function and protein profiles after complete global ischemia with anoxia in rat hearts. Hearts were exposed to global ischemia and anoxia (CGI) for 30 or 60 minutes at 37°C, and myofibrils were prepared for measurement of Ca²⁺-dependent Mg²⁺-ATPase activity at pH 7.0 and 6.5. Hearts incubated in cold saline (1±1°C) and nonincubated hearts served as controls. Maximum ATPase activity was unchanged at pH 7.0 and pH 6.5 in myofibrils from hearts treated with 30 or 60 minutes of CGI. At pH 7.0, the Hill coefficient, which is an index of cooperative interactions among thin-filament proteins, was unchanged after 30 minutes of CGI but was significantly increased after 60 minutes of CGI. A similar trend for increased cooperativity was observed when myofibrillar ATPase activity was measured at pH 6.5 in myofibrils from rat hearts made ischemic for 30 or 60 minutes. Both 30 and 60 minutes of CGI resulted in increased pCa₀ values (half-maximally activating free [Ca²⁺]) at pH 7.0 and pH 6.5. Densitometric analysis of myofibrillar proteins separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated that troponin I and troponin T were degraded during 60 minutes of CGI. Two new protein bands appearing in ischemia-treated myofibrils were identified as partially degraded troponin I and troponin T with Western blots. The troponin I fragment could be phosphorylated by cAMP-dependent protein kinase. In addition, we observed phosphorylation of a protein band that corresponded to myosin light chain-2 in myofibrils from CGI-treated hearts. These results suggest that degradation of thin-filament proteins may contribute to the changes in cooperativity of Ca²⁺ regulation of ATPase activity observed in the myofibrils from rat hearts exposed to CGI. (Circulation Research 1992;70:302–313)

Myocardial ischemia often causes diminished cardiac function. Damage to myofibrillar proteins may be one component contributing to the resulting injury that develops with ischemia. Previous investigators have measured myofibrillar ATPase activity using various animal models of myocardial ischemia to show that ischemia alters the inherent function of the contractile apparatus. In a few studies using coronary artery ligation, degradation of specific myofibrillar proteins was observed, although the identity of various protein fragments was uncertain. Evidence for changes in the composition and function of specific myofibrillar proteins has not been examined in globally ischemic rat hearts.

Myofibrillar proteins have been measured in human serum after an ischemic episode or myocardial infarction. Fragments of myosin heavy chains were detected in patients with acute myocardial infarctions, yet peak levels were not achieved until several days after infarction. Although myosin release closely correlated with infarct size, it was not a good indicator for the onset of injury because of the lengthy time interval required before detection of elevated myosin. In contrast, the appearance of the myosin light chains (MLCs) in plasma correlated well with the onset and severity of acute myocardial infarction. Tropomyosin (Tm), troponin T (TnT), and troponin I (TnI) also were detectable within hours after the onset of infarction. However, the correlation between serum Tm or TnT levels and infarct size has not been examined. Release of TnI did not always correlate well with infarct size. Although the appearance of myofibrillar proteins in serum indicates that these proteins were degraded within the heart during myocardial ischemia, these studies provide little information about changes in
myofibrillar proteins and their inherent function within the heart.

The purpose of our study was to characterize changes in myofibrillar function in hearts exposed to complete global ischemia and to determine whether specific components of the contractile apparatus are altered such that they change myofibrillar function. Myofibrillar function was assessed biochemically by assaying for Ca$^{2+}$-dependent Mg$^{2+}$-ATPase activity. Changes in myofilament components also were examined with emphasis on thin-filament regulatory proteins.

**Materials and Methods**

**Preparations**

The autolysis model described by Jennings and colleagues was used to produce anoxia accompanied by a large, uniform area of complete global ischemia. In their experiments with dog hearts, a 60-minute in vitro incubation of tissue slices at 38°C under anaerobic conditions produced cellular injury that was comparable to 15 minutes of severe, but reversible, in vivo ischemia produced by ligation of the circumflex artery. In our experiments, hearts were rapidly excised from fed, male Sprague-Dawley rats (300–350 g) killed by decapitation. The ventricles were trimmed free of excess tissue, rinsed in cold saline, and divided into four groups: hearts immediately used for preparation of myofibrils (nonincubated control [NIC]), hearts used for preparation of myofibrils and subsequently heated at 100°C for 10 minutes (heat-treated control [HC]), hearts placed in cold saline (1°C±1°C) for 30 or 60 minutes (saline control [SC]), and hearts exposed to 30 or 60 minutes complete global ischemia with anoxia (CGI) at 37°C before the preparation of myofibrils. CGI was produced by placing isolated tissue in impermeable plastic bags (Ziploc) with a small amount of saline (<2.5 ml). Air and excess saline were expressed from each bag, and samples were placed in a 37°C water bath for 30 or 60 minutes.

Rat weight, heart weight after treatment (data not shown), and protein yield (30-minute NIC, 15.87±2.57 [mean±SEM] mg/g wet weight, n=12; 30-minute SC, 13.27±1.60 mg/g wet weight, n=8; 30-minute CGI, 14.16±3.61 mg/g wet weight, n=10; 60-minute NIC, 16.96±0.78 mg/g wet weight, n=10; 60-minute SC, 16.24±1.17 mg/g wet weight, n=6; 60-minute CGI, 14.48±1.75 mg/g wet weight, n=9) were not different among the control and CGI treatment groups. NIC and SC-treated myofibrils were prepared over the same time frame as the corresponding CGI-treated myofibrils. Thus, 30-minute NIC and 60-minute NIC refer to control myofibrils prepared over the same time interval as 30-minute and 60-minute CGI-treated myofibrils, respectively.

After the experimental interval, hearts were weighed, and myofibrils were prepared as described by Pagani and Solaro. Two hearts were used for each myofibrillar preparation. Rat myofibrillar preparations were assayed for protein concentration and for myofibrillar Ca$^{2+}$-ATPase activity the next day. In most experiments, inhibitors of proteolysis were present throughout the isolation of myofibrils (0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride). These inhibitors did not alter the biochemical or gel electrophoresis profiles in any of the myofibril preparations. For gel and Western blot experiments, standards were from bovine cardiac TnT and TnI isolated using the procedure of Potter.

**Procedures**

Protein concentration was determined using a modified Lowry protein assay described by Pagani and Solaro. The protocol of Blanchard and Solaro was used to assay myofibrillar actomyosin ATPase (Ca$^{2+}$-stimulated Mg$^{2+}$-ATPase). Inorganic phosphate production during the ATPase assay was determined using the method of Carter and Karl. Calcium concentrations ranged between pCa values of 4.875 and 8.0 for myofibrillar ATPase activity assays carried out at pH 7.0 and 6.5.

Myofibrillar proteins were separated with sodium dodecyl sulfate (SDS) and 12.5% or 10.5% polyacrylamide gels with the method of Laemmli. Alkaline urea gels were used to identify possible changes in troponin C (TnC) after CGI. Urea and SDS gels were stained with Coomassie blue, and duplicate 10.5% SDS–polyacrylamide gels were transferred to nitrocellulose sheets at 85 mA for 65–70 minutes at 4°C as described by Towbin et al. After protein transfer, nitrocellulose was incubated in Tris-buffered saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween-20) containing 7.5% nonfat dry milk to prevent subsequent nonspecific antibody (Ab) binding. The nitrocellulose was then incubated with buffer containing monoclonal mouse anti-TnT Ab (TnT mAb, JLT-12 clone, diluted 1:200, Sigma Chemical Co., St. Louis, Mo.) or polyclonal rabbit anti-TnI Ab (TnI pAb, diluted 1:500) prepared as described by Dieckman and Solaro. Western blots for actin were also performed using mAbs (B4, HUC1-1, diluted 1:200) from Dr. J. Lessard, University of Cincinnati. Alkaline phosphatase–linked Ab from Promega Corp., Madison, Wis., was added to allow detection of TnT, actin, or TnI (TnT and actin, anti-mouse mAb; TnI, anti-rabbit mAb; diluted 1:7,500). Color development was carried out in development buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 5 mM MgCl$_2$) containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (ProMega). Stained gels and nitrocellulose immunoblots were scanned with a 2222-020 Ultra-Scan XL laser densitometer (LKB Produkter AB, Bromma, Sweden). Peaks identified on the densitometer scans were integrated using GELSCAN software (LKB) and/or with gravimetric methods.

Incorporation of $^{32}$P into myofibrillar proteins in the presence of CAMP-dependent protein kinase A (cAMP-PKA) was measured using methods previously described by our laboratory. The reaction was
initiated by adding [32P]ATP (final concentration, 10 μCi/ml in 0.5 mM ATP; Amersham Corp., Arlington Heights, Ill.) and then stopped at 0, 10, 20, or 30 minutes with cold 10% trichloroacetic acid containing 0.4% pyrophosphate. Samples were filtered through a 0.45-μm HA filter (Millipore Corp., Bedford, Mass.) by suction and washed four times. Radioactivity remaining on the filter was counted in a Tri-Carb 2200CA liquid scintillation counter (Packard Instrument Co., Inc., Meriden, Conn.). The amount of phosphorylation was determined from the plateau portion of the 32P incorporation curve.

Autoradiography was done to determine whether there were changes in the phosphorylation of individual myofibrillar proteins. Myofibrils labeled with [32P]ATP for 30 minutes were placed on ice and then dialyzed against SDS. Dialyzed samples were run on 12.5% acrylamide SDS-polyacrylamide gels, stained with Coomassie blue, and then placed in intensifying cassettes containing Kodak X-OMat XRP-1 film. The density of phosphorylation was analyzed using the LKB densitometer and GELSCAN software.

Statistics

Results shown in all figures and tables are mean±SEM. Results from myofibrillar ATPase activity measurements were normalized based on maximal ATPase activity. Normalized ATPase values were fit to the Hill equation using a nonlinear regression analysis of the Hill equation (GRAPHPAD, ISI Software, version 2.0) to derive the pCa50 and Hill coefficient (Hill n). Comparison of control and CGI-treated rat heart myofibrillar ATPase activity was carried out using Student’s unpaired t test. Protein isolation data, densitometer analyses, and phosphorylation measurements were compared using a one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test. A value of p<0.05 was considered significant for all comparisons.

Results

The functional activity of myofibrils was assessed from maximum Ca2+-stimulated Mg2+-ATPase activity, the pCa50, and the Hill n. The pCa50 reflects myofibrillar Ca2+ sensitivity; the Hill n is a measure of cooperative interactions among thin-filament proteins, either of which may be affected by ischemia. At pH 7.0 these values for myofibrils from 30-minute SC-treated or 60-minute SC-treated rat hearts were not significantly different from those of NIC rat hearts (data not shown). Thus, NIC and SC values for each incubation period (30 versus 60 minutes) were pooled for comparison to CGI-treated rat heart myofibrils (Figures 1A and 2A). Maximum myofibrillar ATPase activity and the Hill n for Ca2+ activation at pH 7.0 were not altered from control values by 30 minutes of CGI (Figure 1A). Maximum ATPase activity also was unchanged after 60 minutes of CGI (Figure 2A). In contrast, a significant leftward shift in the pCa50 was present after 30 and 60 minutes of CGI (Figures 1A and 2A), and 60 minutes of CGI was associated with a significant increase in the Hill n at pH 7.0 compared with control values (Figure 2A).

Additional assays were carried out at pH 6.5, inasmuch as pH decreases during ischemia and acidic pH alters the Ca2+ sensitivity of myofibrils via effects on thin-filament proteins.2,6 Results from
Figure 2. Graphs showing myofibrillar Ca\textsuperscript{2+}-dependent Mg\textsuperscript{2+}-ATPase activity at pH 7.0 (panel A) and pH 6.5 (panel B) in preparations from control rat hearts and from rat hearts exposed to 60 minutes of complete global ischemia with anoxia (60-minute CGI). Values shown are mean ± SEM of percent maximum ATPase activity. Results pooled from nonincubated control and 60-minute saline–treated control rat heart myofibrils incubated at pH 7.0 (panel A, ●) were as follows: maximum ATPase, 121.89 ± 8.69 nmol/mg/min; pCa\textsubscript{50}, 6.05 ± 0.01; and Hill coefficient, 2.01 ± 0.05 (n = 9, r\textsuperscript{2}=0.99). In myofibrils from 60-minute CGI–treated rat hearts incubated at pH 7.0 (panel A, ○) results were as follows: maximum ATPase activity, 131.65 ± 32.07 nmol/mg/min; pCa\textsubscript{50}, 6.20 ± 0.02 (p < 0.05 vs. control); and Hill coefficient, 2.33 ± 0.12 (p < 0.05 vs. control) (n = 5, r\textsuperscript{2}=0.99). Composite results from nonincubated control and 60-minute saline–treated control rat heart myofibrils incubated at pH 6.5 (panel B, ●) were as follows: maximum ATPase, 112.78 ± 6.91 nmol/mg/min; pCa\textsubscript{50}, 5.86 ± 0.03; and Hill coefficient, 2.16 ± 0.31 (n = 7, r\textsuperscript{2}=0.97). When myofibrils from 60-minute CGI–treated rat hearts were incubated at pH 6.5 (panel B, ○), results were as follows: maximum ATPase activity, 109.77 ± 4.66 nmol/mg/min; pCa\textsubscript{50}, 6.01 ± 0.02 (p < 0.05 vs. control); and Hill coefficient, 2.56 ± 0.37 (n = 5, r\textsuperscript{2}=0.98). Composite control values were compared with 60-minute CGI–treated myofibrillar ATPase values at each pH using an unpaired Student’s t test with p < 0.05 considered significant.

Figure 3. Representative densitometer scans of gels containing 50 and 100 μg dialyzed myofibrillar protein. NIC, nonincubated control rat hearts; 60 min SC, rat hearts placed in cold saline for 60 minutes; 60 min CGI, rats hearts exposed to 60 minutes of complete global ischemia with anoxia. Scans were done between myosin and troponin T for each lane. No differences were found among the three different groups.

The two groups of myofibrils from SC hearts (30 and 60 minutes) incubated at pH 6.5 were combined with corresponding NIC values (Figures 1B and 2B) because maximum ATPase, pCa\textsubscript{50}, and Hill n values were not significantly different (data not shown). As reported previously by our laboratory,\textsuperscript{13} acidic pH caused a rightward shift in the pCa\textsubscript{50} and increased the Hill n but had no effect on maximum ATPase activity in control myofibrils (Figure 1A versus 1B, Figure 2A versus 2B). Acidic pH was also associated with a decrease in the pCa\textsubscript{50} for activation of myofibrils from 30-minute CGI–treated (Figure 1A versus 1B) and 60-minute CGI–treated (Figure 2A versus 2B) hearts. This shift in pCa\textsubscript{50} was similar to the shift observed in control myofibrils (Figure 1A versus 1B, Figure 2A versus 2B). The Hill n also tended to increase in the 30-minute CGI–treated (Figure 1B) and 60-minute CGI–treated (Figure 2B) rat heart myofibrils incubated at pH 6.5 compared with control values. However, at both time points the increase in the Hill n at pH 6.5 did not reach statistical significance (Figures 1B and 2B). Overall, it appears that myofibrillar protein functions or conformational changes involved in the pCa shift caused by acidic pH are not appreciably affected by 60 minutes of complete in vitro global ischemia.

To determine whether the changes in ATPase activity were associated with the breakdown of individual proteins, we analyzed the myofibrils from ischemic hearts using gel electrophoresis. The protein profile between myosin and TnT was analyzed in NIC, 60-minute SC–treated, and 60-minute CGI–treated rat heart myofibrils and was not different when 50 μg or 100 μg protein was loaded onto gels (Figure 3). Gel proteins between actin and MLC-2 also were analyzed after 30- and 60-minute CGI and then compared with control profiles in Figures 4A and 5A, respectively. The protein profiles from NIC (Figures 4B and 5B) and 30-minute SC–treated (Figure 4B) or 60-minute SC–treated (Figure 5B)
myofibrils were not different. After 30 minutes of CGI, a new protein band appeared just above MLC-2 (Figure 4A, arrow). This band remained prominent after 60 minutes of ischemia (Figure 5A). In addition, a second protein band was observed between TnT and Tm in hearts exposed to 60 minutes of CGI (Figure 5A). Both bands were evident on densitometer scans of gels (Figures 4 and 5). The protein band present after 30 and 60 minutes of CGI corresponds to a band observed by Toyo-Oka and Ross3 in dogs exposed to coronary artery ligation for 6 hours. Gravimetric analysis also indicated that 60 minutes of

![Image](http://circres.ahajournals.org/)

**Figure 4.** Representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, panel A) and densitometer scans of gels (panel B) from rat heart myofibrillar preparations. Tn, troponin; Tm, tropomyosin; MLC, myosin light chain; Control, nonincubated control rat hearts; 30 min CGI, rat hearts exposed to 30 minutes of complete global ischemia with anoxia; Saline Control, 30-minute saline-treated rat hearts. A 12.5% acrylamide separating gel and 4.5% acrylamide stacking gel were used to observe thin-filament proteins. Each lane in panel A was loaded with 150 μg SDS-dialyzed myofibrillar protein from the following treatment group: Control, lane 1; 30 min CGI, lane 2; and Saline Control, lane 3. Peaks shown in panel B include the following: 1, actin; 2, TnT; 3, Tm; 4, Tn; 5, MLC-1; and 6, MLC-2.

![Image](http://circres.ahajournals.org/)

**Figure 5.** Representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, panel A) and densitometer scans of gels (panel B) from rat heart myofibrillar preparations. Tn, troponin; Tm, tropomyosin; MLC, myosin light chain; Control, nonincubated control rat hearts; 60 min CGI, rat hearts exposed to 60 minutes of complete global ischemia with anoxia; Saline Control, 60-minute saline-treated rat hearts; Heat-treated Control, rat hearts heated at 100°C for 10 minutes. Gels were prepared and run as in Figure 4A. Each lane in panel A contains the following treatment group: Saline Control, lane 1; Heat-treated Control, lane 2; Control, lanes 3 and 4; and 60 min CGI, lane 5. Arrows in panels A and B correspond to new peptide bands observed in myofibrils from CGI–treated hearts. Peaks shown in panel B are identified using the same numbering described in Figure 4B.
TABLE 1. Densitometer Analysis of Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis of Myofibrils From Control Rat Hearts, From Rat Hearts Exposed to 60 Minutes of Complete Global Ischemia With Anoxia, and From Heat-Treated Rat Hearts

<table>
<thead>
<tr>
<th>Protein band</th>
<th>Control (n=10)</th>
<th>CGI (n=5)</th>
<th>Heat-treated (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin+troponin T</td>
<td>45.86±0.75</td>
<td>47.04±2.36</td>
<td>46.96±2.04</td>
</tr>
<tr>
<td>Actin*</td>
<td>39.10±0.52</td>
<td>42.28±2.89</td>
<td>42.82±1.45</td>
</tr>
<tr>
<td>Troponin T*</td>
<td>7.15±0.67</td>
<td>3.78±0.61†</td>
<td>4.38±0.62</td>
</tr>
<tr>
<td>New band 1</td>
<td>0.05 (1)</td>
<td>1.66±0.40†</td>
<td>ND</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>15.06±0.24</td>
<td>13.75±0.25</td>
<td>14.07±0.52</td>
</tr>
<tr>
<td>Troponin I</td>
<td>9.11±0.36</td>
<td>5.59±0.31†</td>
<td>7.18±0.95</td>
</tr>
<tr>
<td>MLC-1</td>
<td>15.07±0.32</td>
<td>14.24±1.11</td>
<td>15.06±2.44</td>
</tr>
<tr>
<td>New band 2</td>
<td>0.15±0.10</td>
<td>3.43±0.54†</td>
<td>ND</td>
</tr>
<tr>
<td>MLC-2</td>
<td>15.79±0.38</td>
<td>14.16±0.97</td>
<td>16.80±1.54</td>
</tr>
<tr>
<td>Total protein (relative area [UI])</td>
<td>0.1957±0.0063</td>
<td>0.1903±0.0121</td>
<td>0.1947±0.0025</td>
</tr>
</tbody>
</table>

Values are mean±SEM and were calculated as a percentage of total protein integrated gravimetrically between actin and myosin light chain (MLC)-2. Control, pooled values from nonincubated control and 60-minute saline–treated control rat hearts; CGI, rat hearts exposed to 60 minutes of complete global ischemia with anoxia; Heat-treated, rat heart myofibrils heated at 100°C for 10 minutes; n, number of myofibrillar preparations; ND, densitometer unable to detect any significant peak. Scans were obtained from gels containing 150 μg protein per lane. Parentheses indicate number of myofibrillar preparations. Results were compared using a one-way analysis of variance with unequal replication and a post hoc Newman–Keuls test.

*Actin and troponin T bands overlapped; thus, these values are estimates of actin and troponin T content.

**p<0.05 vs. control values.

CGI resulted in a significant reduction of TnT and TnI when normalized to total protein scanned in a region of the gel between actin and MLC-2 (Table 1). Similar results were obtained with the exception of TnT and actin when protein bands were integrated with LKB GELSCAN software (data not shown). Overlap of the TnT and actin bands made computer integration of the two peaks difficult. The amount of protein loaded onto each gel did not alter these findings (Figure 6). Western blots for actin verified that actin was not degraded (Figure 7). Analysis with alkaline urea gels indicated TnIc also was not degraded in myofibrils from 60-minute SC–treated or 60-minute CGI–treated hearts compared with NIC hearts (Figure 8). When myosin was separated by SDS gel electrophoresis and analyzed densitometrically, it also was not degraded in 60-minute CGI–treated hearts (Figure 9).

Although proteolytic inhibitors present during the preparation of myofibrils did not affect the gel protein profile, nonspecific proteolysis could have occurred during CGI and could have given rise to the two new protein bands. Heat treatment (100°C) of isolated myofibrils indicated that very little nonspecific degradation of proteins occurred during CGI (Figure 5, Table 1). The profile of HC myofibrils on gels was similar to controls (Figure 5), with no reduction in TnI and only a small reduction in TnT. There were no significant proteins migrating with the same mobility as the two bands found in myofibrils from CGI–treated rat hearts.

Toyo-Oka and Ross suggested that degradative products of TnI, TnT, and TnC appeared on gels prepared from dog hearts exposed to ligation of the circumflex artery. However, they were unable to identify the new protein bands. To determine whether the two new protein bands appearing on our gels were proteolytic products of thin-filament proteins, we analyzed the preparations using immunoblots (Figures 10 and 11). No additional protein
bands were observed on Western blots for actin (Figure 6). Protein band 1 (Table 1), which appeared with 60 minutes (Figure 5A) but not 30 minutes of CGI (Figure 4A) was identified as a product of TnT digestion on Western blots probed with a TnT mAb (Figure 10A) and analyzed with densitometry (Figure 10B). This product was not apparent in NIC or SC samples. The second proteolytic protein product (band 2, Table 1) was identified as a Tnl product with a Tnl pAb (Figure 11). The percentage of protein appearing in the TnT and Tnl fragments was comparable to the percent reduction in TnI and TnT bands. Thus, neither TnT nor Tnl appeared to be completely degraded during 60 minutes of CGI. The presence of a Tnl fragment agrees with the predictions of Toyo-Oka and Ross, who used coronary artery ligation in dogs. To gain more information about this peptide fragment we did phosphorylation studies. Inotropic drugs acting via cAMP are known to phosphorylate Tnl. The total amount of 32P incorporated over 30 minutes was not significantly changed (p>0.05, one-way ANOVA) in myofibrils isolated from 60-minute CGI-treated rat hearts (1.137±0.158 nmol/mg protein, n=5) compared with NIC hearts (1.268±0.142 nmol/mg protein, n=6) or 60-minute SC hearts (0.908±0.079 nmol/mg protein, n=5).

Autoradiography was done to determine whether phosphorylation of Tnl and/or other myofibrillar proteins changes after exposure to CGI. Very little protein was phosphorylated in control preparations incubated without cAMP-PKA (Figure 12A), which indicates that the majority of phosphorylation was due to the specific actions of cAMP-PKA. Our laboratory has previously identified the phosphorylated proteins as C protein and Tnl,21 and both proteins appear to be phosphorylated to the same extent in NIC and 60-minute SC-treated myofibrils (Figure 12B). A third small peak of phosphorylated protein also was present in both NIC and 60-minute SC-treated myofibrils as a result of autophosphorylation of cAMP-PKA (Figure 12B, arrow b).22 These same three bands also were phosphorylated in myofibrils from CGI-treated rat hearts. However, several new phosphorylated bands were observed in the presence of cAMP-PKA but not in its absence (data not shown). Band d in Figure 12B migrated with MLC-1; band e corresponded to the proteolytic product of Tnl on the gels (Figure 5A). The most prominent of the new bands (band f) migrated in the same position as MLC-2 on gels. Although the band could represent phosphorylation of MLC-2 in CGI-treated hearts, we cannot rule out the possibility that this phosphorylated band was a fragment from other degraded myofibrillar proteins.

Discussion

The results of the present study have shown evidence of thin-filament protein degradation (Figure 5) despite an increased Ca2+ sensitivity (pCa90) and cooperativity (Hill n, Figure 2) in myofibrils from rat hearts exposed to CGI. Continued loss of myofibrillar proteins would be expected to result in the diminished response of ATPase activity to Ca2+ as described in prolonged coronary artery ligation models of ischemia.1,2 Loss of inherent myofibrillar function along with destruction of sarcoplasmic reticulum and sarcosomal function may contribute to loss of myocardial function and the onset of cell death during the course of ischemia.

Myofibrillar function at pH 7.0 was only minimally affected in rat hearts exposed to 30 minutes of CGI. An increase in the pCa90 was observed after 30 minutes of CGI (Figure 1A), and the rightward shift in the pCa90 caused by acidic pH also was not altered.

![Figure 7](attachment:image.png)

**Figure 7.** Panel A: Representative Western blot for actin. Dialyzed myofibrils from each treatment group were run on 12.5% acrylamide minigels (11 μg protein per lane). Proteins were transblotted onto nitrocellulose at 85 mA for 80 minutes at 4°C. Blots shown were stained with the anti-actin monoclonal antibody B4 followed by anti-mouse monoclonal antibody linked to alkaline phosphatase as described in “Materials and Methods.” Panel B: Densitometer scans and corresponding integration patterns shown for rat heart myofibrils. NIC, nonincubated control rat hearts; 60 min SC, rat hearts placed in cold saline for 60 minutes; 60 min CGI, rat hearts exposed to 60 minutes of complete global ischemia with anoxia; AU, absorbance units. Each lane in panel A contains myofibrillar protein from the following treatment groups: 60 min CGI, lane 1; 60 min SC, lane 2; and NIC, lane 3. In panel B, there were no differences in the actin content when the blot was analyzed by densitometry. Similar results were obtained with the HUC1-1 actin antibody.
by 30 minutes of CGI (Figure 1). These results were in general agreement with those of Krause and Hess\textsuperscript{23} using 30 minutes of complete in situ ischemia in dog hearts. Apparently, little breakdown of myofibrillar protein occurred during the 30-minute interval of CGI. Other investigators also have observed minimal evidence of cellular damage in slices of dog hearts exposed to a 30-minute interval of CGI.\textsuperscript{24} Instead, the development of acidic pH during 30 minutes of CGI and the subsequent decrease in myofibrillar Ca\textsuperscript{2+} sensitivity was postulated to contribute to diminished tension development during severe ischemia.\textsuperscript{19,20,23} Indeed, acidosis alone shifted the tension/pCa relation to the right and decreased maximum force in skinned cardiac muscle preparations.\textsuperscript{20,25} Low pH partially reduced tension and myofibrillar ATPase activity by inhibiting Ca\textsuperscript{2+} binding to TnC,\textsuperscript{13,20} but the explanation for reduced maximum force has remained unclear. Acidic pH also can alter other cellular processes that could diminish contractile function\textsuperscript{20} in addition to its effects on myofibrils.

As with pCa\textsubscript{50} values after 30 minutes of CGI, a significant increase in the pCa\textsubscript{50} was observed after 60 minutes of CGI. Exposure to 60 minutes of CGI moderately increased the myofibrillar Hill n (Figure 2A). These results indicate that prolonged ischemia slightly increased myofibrillar sensitivity to Ca\textsuperscript{2+} and enhanced cooperative Ca\textsuperscript{2+} activation of the actin–crossbridge reaction. Myofibrillar function after 60 minutes of CGI has not been reported for rat hearts; however, myofibrillar ATPase measurements have been made in rat\textsuperscript{1} and dog\textsuperscript{2} hearts made ischemic by chronic coronary artery ligation. In contrast to our results, coronary artery ligation generally decreased maximum ATPase activity\textsuperscript{1} and/or diminished sensitivity of myofibrils to Ca\textsuperscript{2+}.\textsuperscript{2} Katagiri\textsuperscript{2} reported a slight initial increase in the maximum ATPase activity of myofibrils prepared from dog hearts with an occluded left anterior descending coronary artery.

**Figure 8.** Alkaline urea gel for troponin C. The separating gel contained 12.5% acrylamide, and the stacking gel contained 4.5% acrylamide. Each lane was loaded with 150 μg protein, and each lane contains urea-dialyzed myofibrils from the following treatment groups: bovine standard, lane 1; nonincubated control rat hearts, lane 2; rat hearts exposed to 60 minutes of complete global ischemia with anoxia, lane 3; and rat hearts placed in cold saline for 60 minutes, lane 4. Arrow indicates location of troponin C on gels.

**Figure 9.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and densitometer scans for myosin. Panel A: Sodium dodecyl sulfate–dialyzed myofibrils from nonincubated control rat hearts (NIC, lane 1), from rat hearts placed in cold saline for 60 minutes (60 min SC, lane 2), and from rat hearts exposed to 60 minutes of complete global ischemia with anoxia (60 min CGI, lane 3) (3 μg protein per lane) run on 8.25% acrylamide gels. Panel B: Densitometer scans of gel shown in panel A. AU, absorbance units. Each lane and the corresponding integration pattern are shown and indicate that there were no differences among the groups analyzed.
This was followed by a reduction in activity between 48 hours and 7 days. Similar reductions in maximum myofibrillar ATPase activity have been observed at 3 and 11 weeks after ligation of the left anterior descending coronary artery in dogs.\(^1\) Decreased Ca\(^{2+}\) sensitivity of myofibrillar superprecipitation has been noted between 6 and 24 hours after coronary artery occlusion in dog hearts.\(^3,26\) However, Ca\(^{2+}\) sensitivity using superprecipitation is difficult to compare with that observed with ATPase measurements.\(^27\) The

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**Figure 10.** Troponin T Western blots and densitometry. Panel A: Representative Western blot for troponin T. Gels containing 10.5% polyacrylamide were loaded with 20–25 µg sodium dodecyl sulfate–dialyzed myofibrillar protein and transblotted onto nitrocellulose at 85 mA for 70 minutes. A mouse monoclonal antibody to troponin T followed by anti-mouse monoclonal antibody linked to alkaline phosphatase was used to detect troponin T as described in “Materials and Methods.” Each lane contains the following treatment groups: bovine standard (Tn-T STD), lane 1; nonincubated control rat hearts (Control), lane 2; rat hearts exposed to 60 minutes of complete global ischemia with anoxia (60 min CGI), lane 3; and rat hearts placed in cold saline for 60 minutes (Saline Control), lane 4. Panel B: Densitometer scans of Western blots shown in panel A.

**Figure 11.** Troponin I Western blots and densitometry. Panel A: Representative Western blot for troponin I. Gels containing 10.5% polyacrylamide were loaded with 40–45 µg sodium dodecyl sulfate–dialyzed myofibrillar protein and transblotted onto nitrocellulose at 85 mA for 65 minutes. A rabbit polyclonal antibody to troponin I followed by anti-rabbit monoclonal antibody linked to alkaline phosphatase was used to detect troponin I as described in “Materials and Methods.” Each lane contains myofibrillar protein from the following treatment groups: bovine standard (Tn-I STD), lane 1; nonincubated control rat hearts (Control), lane 2; rat hearts exposed to 60 minutes of complete global ischemia with anoxia (60 min CGI), lane 3; and rat hearts placed in cold saline for 60 minutes (Saline Control), lane 4. Panel B: Densitometer scans of Western blots shown in panel A.
species, duration, and severity of ischemia may account for differences between our results and those of earlier investigators. These differing results suggest that a continuum of changes in myofibrillar function may develop during the course of ischemia.

Myocardial ischemia may cause changes in individual myofibrillar proteins that affect myofibrillar function. However, previous studies in our laboratory have shown that myosin ATPase activity in dog hearts was not affected by CGI.

As predicted by ATPase measurements, myofibrillar proteins were affected by ischemia. After 60 minutes of CGI, semiquantitative analysis of gels with densitometry indicated that TnI and TnT were degraded (Table 1), and products of TnT and TnI proteolysis were identified using Western blots (Figures 10 and 11). Protein measurements and densitometry indicated that myofibrillar protein yield was unchanged after ischemia. However, it is possible that soluble peptide fragments were lost during myofibrillar preparation, which would result in underestimation of changes in myofibrillar structure.

Only a few of the structural changes that may occur in myofibrillar proteins during ischemia have been explored in previous studies, and most of the changes have been examined in models using coronary artery ligation. Although some investigators reported no change in myofibrillar protein concentrations after coronary artery ligation, the majority reported decreases in some proteins. Katagiri found reductions in myosin and α-actinin after 3 days of coronary artery ligation in dogs, whereas shifts in myosin isoforms were observed 3 weeks after ligation. With our model, separation of myosin on 8.25% acrylamide gels indicated that its content was not reduced after 60 minutes of CGI (Figure 9). Decreases in some thin-filament protein concentrations, including TnC, TnT, and TnI, have been observed within 6 hours of ligation. Yet, TnC did not appear to be degraded in our samples when analyzed with alkaline urea gels (Figure 5). This indicates that TnC degradation may occur at a later point in time than proteolysis of other thin-filament proteins. An additional protein peak below MLC-2 was described by Toyo-Oka and Ross, but we did not observe prominent new peaks below MLC-2 in myofibrils from CGI-treated hearts. A relatively long time course of ischemia may be required to detect this band, and/or the interval when this protein appeared may not have been used during our study.

Maintenance of TnC content during ischemia and increased cooperativity among thin-filament proteins indicates that proteolysis of TnI and/or TnT may contribute to changes in myofibrillar function that
result from 60 minutes of CGI. The peptide resulting from TnT degradation (Figures 5 and 10) has not been observed by previous investigators. This TnT peptide product may not appear in other species, or it may appear only transiently during coronary artery ligation in the dog. Degradation of TnT and the appearance of a slightly smaller product may have contributed to the increased cooperativity and slight increase in Ca\(^{2+}\) sensitivity observed in myofibrils from CGI-treated hearts. Cleavage of amino acids from either end of the TnT protein could influence myofibrillar function. Various skeletal\(^{29}\) and cardiac\(^{30,31}\) TnT isoforms, which vary in the length and sequence of amino acids near the N-terminus, have been associated with altered troponin–Ca\(^{2+}\) binding sensitivity\(^{30,31}\) and cooperativity.\(^{20}\) Less is known about how removal of amino acids from the carboxy terminal site would affect cardiac TnT function. Investigators\(^{32,33}\) have shown that a portion of the Ca\(^{2+}\)-sensitive Tm binding site lies within the last 10 amino acid residues in rabbit skeletal muscle TnT. In addition, TnI\(^{32,34}\) and TnC\(^{32,35}\) binding sites have been found in the carboxy terminus of skeletal TnT. Similar amino acid sequences are observed in rat cardiac\(^{36}\) and skeletal\(^{37}\) TnT, which suggests that similar binding sites exist in cardiac TnT. Loss of carboxy terminal amino acids during CGI could affect one or all of these binding sites and thus alter the Ca\(^{2+}\) sensitivity of the thin filament.

TnI was a second component that may have contributed to changes in myofibrillar function. The peptide identified as a proteolyzed TnI peptide (Figure 11) was apparently also observed with an autolysis model\(^{28}\) and with coronary artery ligation\(^2\) in dog hearts. The peptide released from TnI during ischemia retained some substrate activity for phosphorylation by cAMP-PKA (Figure 12). In contrast, \(\beta\)-adrenergic–stimulated TnI phosphorylation decreased in isolated, perfused rat hearts made ischemic for 60 minutes,\(^{38}\) and reperfusion of the same preparation resulted in complete loss of \(\beta\)-adrenergic–mediated TnI phosphorylation. Although Bartel et al\(^{38}\) did not observe a TnI peptide fragment similar to one described in the present study, perfusion was performed during the interval of ischemia, which may have resulted in washout of the TnI fragment and a subsequent reduction in cAMP-dependent phosphorylation of TnI. Since the preferred site for cAMP-mediated phosphorylation is near the N-terminus of TnI,\(^{39,40}\) the fragment of TnI observed in our gels may include the N-terminus of TnI (Figure 11). In addition, the enhanced Hill n and increased pCa\(_{50}\) observed in ischemic preparations indicate that the TnI sequence known to inhibit actomyosin ATPase activity (residues 130–150) was most likely not cleaved during ischemia. More specific insight into the amino acids cleaved from TnI and TnT during CGI and the contribution made to changes in myofibrillar ATPase Ca\(^{2+}\) sensitivity and cooperativity require further study.

In conclusion, our investigation found that myofibrillar proteins were already being degraded during CGI, although myofibrillar function was only minimally affected. Reduced contractility early on in ischemia cannot be attributed to loss of myofibrillar protein degradation, but it may have important functional consequences during more prolonged ischemia or during recovery from an ischemic episode.

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References


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