Decline of contractility during ischemia-reperfusion injury: actin glutathionylation and its effect on allosteric interaction with tropomyosin

Frank C. Chen and Ozgur Ogut

Cardiovascular Contractility and Signaling Laboratory, Division of Cardiovascular Diseases, Mayo Clinic, Rochester, Minnesota

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Chen, Frank C., and Ozgur Ogut. Decline of contractility during ischemia-reperfusion injury: actin glutathionylation and its effect on allosteric interaction with tropomyosin. Am J Physiol Cell Physiol 290: C719–C727, 2006. First published October 26, 2005; doi:10.1152/ajpcell.00419.2005.—The severity and duration of ischemia-reperfusion injury is hypothesized to play an important role in the ability of the heart subsequently to recover contractility. Permeabilized trabeculae were prepared from a rat model of ischemia-reperfusion injury to examine the impact on force generation. Compared with the control perfused condition, the maximum force ($F_{\text{max}}$) per cross-sectional area and the rate of tension redevelopment of $F_{\text{max}}$ with ischemia was accompanied by a decline in fiber ischemia despite the availability of a high concentration of ATP. The reduction in $F_{\text{max}}$ with ischemia was accompanied by a decline in fiber stiffness, implying a drop in the absolute number of attached cross bridges. However, the declines during ischemia were largely recovered after reperfusion, leading to the hypothesis that intrinsic, reversible posttranslational modifications to proteins of the contractile filaments occur during ischemia-reperfusion injury. Examination of thin-filament proteins from ischemic or ischemia-reperfused hearts did not reveal proteolysis of troponin I or T. However, actin was found to be glutathionylated with ischemia. Light-scattering experiments demonstrated that glutathionylated G-actin did not polymerize as efficiently as native G-actin. Although tropomyosin accelerated the time course of native and glutathionylated G-actin polymerization, the polymerization of glutathionylated G-actin still lagged native G-actin at all concentrations of tropomyosin tested. Furthermore, cosedimentation experiments demonstrated that tropomyosin bound glutathionylated F-actin with significantly reduced cooperativity. Therefore, glutathionylated actin may be a novel contributor to the diverse set of posttranslational modifications that define the function of the contractile filaments during ischemia-reperfusion injury. force; troponin; cooperativity

THE OCCLUSION OF AN ARTERY is a primary cause of myocardial ischemia, the physiological condition of low or no blood flow that results in $O_2$ deprivation in the myocardium. This condition may occur in an acute setting as a result of rupture of an atherosclerotic lesion and consequent occlusion due to atherothrombosis (15). The duration and extent of the ischemic period is thought to be a primary determinant of the chance of consequent infarction, with as little as 15 min of proximal coronary occlusion resulting in some degree of irreversible cell injury (28, 33). The interruption of blood flow with coronary occlusion causes rapid consumption of available $O_2$ in affected areas of the myocardium, shifting the cell from aerobic to anaerobic metabolism (28). This condition results in a rapid and large (>40%) decline in ATP and creatine phosphate (CP) content in cardiomyocytes (5, 6, 9, 24, 42, 43, 62) that is readily apparent within 10 min of the onset of ischemia. Because ATP is the substrate for the actomyosin ATPase, its large decline during ischemia suggests that this is a causative event for the decrease in force produced by ischemic myocardium. However, concentrations of ATP during ischemia remain well above the $K_m$ of myosin (9, 14, 43, 62), suggesting that reductions in ATP cannot solely explain the observed decline in the contractility of the heart. Therefore, additional mechanisms are hypothesized to impair force production during ischemia-reperfusion injury.

Studies of the molecular consequences of ischemia-reperfusion injury suggest that specific proteolysis of sarcomeric proteins may play a prominent role (13, 22, 54, 58). Specifically, cleavage of troponin I (TnI), the inhibitory subunit of the troponin complex, was shown to occur at the COOH terminus of the protein. Van Eyk et al. (54) suggested that the observed cleavage of sarcomeric proteins during ischemia-reperfusion injury correlated with a decline in force production along with a change in the Ca$^{2+}$ sensitivity of the muscle fibers. The functional consequences of this protein cleavage have been studied in a transgenic mouse line expressing the truncated TnI protein TnI$_{1,193}$ (34, 41). In addition, specific cleavage of troponin T (TnT), the tropomyosin (Tm)-binding subunit of the troponin complex, may also occur (12), possibly through a caspase-mediated pathway (13). In addition to proteolysis, posttranslational modification of proteins resulting from changes in the cell redox state have been observed during ischemia-reperfusion or other inflammatory injury (19, 20). The formation of reactive oxygen species is postulated as an additional cause of protein damage during ischemia-reperfusion injury, resulting in the oxidation of proteins at susceptible amino acids (1, 2). The impact of proteins modified by reactive oxygen species may be regulated by reversal of the modification (35, 39, 60, 61) or by the protein’s subsequent proteolysis by the proteasome (21, 49). However, it has been established that such modifications are not summarily negative but may play a role in modulating protein activity (31, 32, 56, 63). It is therefore hypothesized that changes in the cell redox state in ischemia-reperfusion injury may target proteins of the actomyosin filaments to regulate contractility during, and recovery from, the ischemic state (19). Such protein targets would be of particular interest, as they might contribute directly to acute changes in myocardial contractility.

To examine contractile function during ischemia-reperfusion injury, permeabilized trabeculae from a rat model were
used to measure force generation in the presence and absence of external load. The results demonstrated a reduction in the maximum force (F_{max}) per cross-sectional area, the rate of tension redevelopment (Ku), and fiber stiffness with ischemia that was not rescued by ATP, suggesting that posttranslational modification of contractile filament proteins may be involved. These changes in contractile function during ischemia were accompanied by the appearance of a glutathionylated form of sarcomeric actin (19, 57). Additional data are provided to suggest that glutathionylation of actin may alter its allosteric interaction with Tm, providing a novel point for further study of the effect of ischemia-reperfusion injury on cardiac muscle thin-filament function.

**MATERIALS AND METHODS**

**Ischemia-reperfusion injury model.** This model was described in detail previously (7). Male Sprague-Dawley rats were anesthetized using intramuscular xylazine, ketamine HCl, and acepromazine (3: 3: 2; 0.5–0.75 ml/kg) in accordance with protocols approved by the Institutional Animal Care and Use Committee. An endotracheal tube was inserted for ventilation with O2-supplemented room air with the use of a positive pressure respirator (5–15 mmHg). Body temperature was maintained at 37°C during the procedure. The heart was exposed by performing a midline thoracotomy, and a ligature was placed around the left anterior descending coronary artery close to its origin from the aorta. The ends were exteriorized and passed through polyethylene tubing. Coronary occlusion was achieved by pressing the tube against the heart muscle while pulling the ligature, followed by clamping the tube with a hemostat. Rfow was initiated by releasing the ligature. Experimental conditions tested included 90-min control perfusion and up to 30 min of left anterior descending coronary occlusion (ischemia), followed by 0 or 60 min of reperfusion. Trabeculae were prepared from the ischemic zone of the left ventricle.

**Contractility measurements.** The procedures and solutions used in the muscle preparations were described previously (45). Trabeculae from the left ventricle were excised, and their ends were fixed using 1% glutaraldehyde in 50% glycerol (10), followed by attachment of aluminum T-clips. Trabeculae were skinned for 60 min at 4°C in pCa9 (1 × 10^{-9} M free Ca^{2+}) solution with 1% Triton X-100 and then transferred to a workstation (45). The trabeculae were stretched to an average sarcomere length of ~2.2 μm (45), and the length of the fiber was recorded as original length. Trabeculae were activated with solutions containing various free Ca^{2+} concentrations ([Ca^{2+}]_o). Force generated was normalized to F_{max} by calculating the elliptical cross-sectional area of the fiber. Experiments were temperature controlled to 37°C ± 0.1°C. Measurements predicated on controlling the length or force of the fiber were programmed using the 600A Controller device (Aurora Scientific, Aurora, ON, Canada). K_{st} experiments were performed as described previously (45). At least six measurements of K_{st} were taken from each fiber. Data regarding force as a function of free Ca^{2+} were fit using the Hill equation to determine the cooperativity of force activation, or Hill coefficient (n_H). The maximum velocity (V_{max}) of shortening was determined by isometric contractions at various percentages of F_{max} (3–100%). Each isometric contraction was performed in triplicate. Velocities were normalized to muscle lengths (ML) per second, plotted as a function of force, and fit by the Hill equation (14, 27) to determine the extrapolated velocity at zero force. Fiber stiffness was measured as described previously (46). Data are presented as averages ± SE. Statistical comparisons between groups were performed using Student’s t-test.

**One- and two-dimensional SDS-PAGE.** For resolution of thin-filament proteins according to M, a 29:1 SDS-PAGE ratio of various acrylamide concentrations was used (46, 47). For two-dimensional PAGE, the muscle was extracted with 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS), 0.5% pH 3–10 immobilized pH gradient (IPG) buffer, 1 mM EDTA, and 1 mM PMSF. Proteins were extracted from Triton X-100-permeabilized muscle strips to ensure that the fiber contraction and resulting protein expression pattern could be correlated reliably. For resolution of actin isolectric variants, appropriate protein amounts from the extracts were added to a solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, and 0.002% bromophenol blue and were allowed to rehydrate pH 4–7 IPG gel strips overnight. After rehydration, proteins were focused in “face-up” mode on an Ettan IPGphor II system (Amersham Biosciences, Piscataway, NJ). After isolectric point separation, the gel strips were equilibrated in 6 M urea, 50 mM Tris-HCl, pH 8.8, 30% glycerol, 2% SDS, and 0.002% bromophenol blue with DTT and iodoacetamide before resolution using M, in the second dimension. After SDS-PAGE was completed, gels were used for Western blotting or were silver stained as described previously (47). To test the reversibility of observed modifications, duplicate isolectric point-focusing experiments were conducted in the presence of DTT, a sulfhydryl-reducing agent.

**Western blot analysis.** PAB antiserum against TnT and TnI (T85 and TnI 88-106, respectively) Fitzgerald Industries International, Concord, MA) or MAb against sarcomeric actin (Sigma, St. Louis, MO) were used for Western blot analysis (47). To test for TnI and TnT proteolysis, three sets of perfused, ischemic, and ischemia-reperfusion homogenates were used. To ensure that proteins cleaved at either the NH2- or COOH-terminal end would be detected reliably, antisera directed against epitopes of the central region of the proteins were used. To detect protein glutathionylation, an anti-glutathione antibody (ViroGen, Watertown, MA) was used. Signals were visualized using alkaline phosphatase substrate development or ECL detection.

**Immunoprecipitation.** Immunoprecipitation of actin was performed with the specific actin antibody and anti-mouse IgM agarose beads (Sigma). An aliquot of homogenate from a permeabilized fiber was dissolved in 1 ml of actin depolymerization buffer (in mM: 2 Tris-HCl, pH 8, 0.2 CaCl2, and 0.2 ATP) and incubated for 30 min at 4°C. The lysate was centrifuged to remove insoluble proteins. Subsequently, 1 μg of actin antibody was added and mixed at 4°C for 2 h, followed by addition of anti-mouse IgM agarose beads and further incubation for 30 min at 4°C. The bead-antibody-antigen complexes were recovered by performing brief centrifugation, washed twice with 10 bead volumes of actin depolymerization buffer, and incubated for 5 min at 60°C in SDS-PAGE sample buffer. The recovered sample was used for Western blot analysis.

**Protein purification.** Actin was purified from rabbit skeletal muscle acto sine powder (48). Polymerization and depolymerization of actin was achieved as described previously (48). Cardiac muscle Tm, predominantly the α-isofom, was purified from chicken heart (Pel-Freeze Biologicals, Rogers, AR) according to a previously published protocol (50). Proteins were quantitated using the NanoOrange protein assay (Molecular Probes, Eugene, OR), as well as absorbance for globular actin (G-actin) with a molar extinction coefficient of 26,460 cm^{-1} M^{-1} (16).

**Actin glutathionylation.** Purified G-actin was glutathionylated using a previously described method (16, 18). A solution of 30 μM G-actin was reacted with a 20-fold excess of 20 mM DTNB in 1% NaHCO3 until an equivalent molar amount of DTNB^- was released as followed using A_{412} nm. This mixture was gel filtered through Sephadex G25 (Sigma), mixed with a 50-fold molar excess of glutathione, and allowed to react for 20 min at room temperature. The final product was gel filtered and then dialyzed against actin depolymerization buffer, followed by a polymerization-depolymerization cycle (48). Glutathionylation was verified using Western blot analysis, and the ratio of glutathionylated to native G-actin was determined by resolution of the two forms using isoelectric focusing, followed by silver...
staining and quantitation using a Personal Densitometer SI and ImageQuant software (Amersham Biosciences). The fraction of glutathionylated G-actin was calculated by dividing the value of the glutathionylated G-actin spot by the sum of the native and glutathionylated G-actin spots. During the preparation of glutathionylated G-actin, the native and glutathionylated G-actin were pooled.

**Light scattering of F-actin.** The polymerization of actin was monitored by measuring the change in light scattering over time (11). Various concentrations of G-actin were dissolved in (in mM) 2 Tris-HCl, pH 8.0, 0.2 CaCl₂, and 0.2 ATP and allowed to equilibrate to 25°C in a Shimadzu RF-5301 spectrofluorophotometer. Polymerization of actin was initiated by the addition of an equal volume of polymerization buffer (in mM: 200 KCl and 4 MgCl₂), followed by light scattering at 360 nm and 1 Hz. Data from three batches of 17%, 36%, and 38% glutathionylated G-actin were pooled. To determine the effect of Tn on actin polymerization, varying concentrations of Tn were dissolved in the polymerization buffer before addition to actin.

**F-actin cosedimentation.** For cosedimentation, 2 μM filamentous actin (F-actin) polymerized from either native G-actin or glutathionylated G-actin was mixed with varying concentrations of Tn in (in mM) 10 Tris-HCl, pH 8.0, 100 KCl, and 2 MgCl₂ (30-μl total volume). The mixture was incubated at 4°C for 2 h and then centrifuged at 100,000 g for 30 min at 4°C. The recovered pellets, along with 25 ng of G-actin and Tm controls, were resolved by performing 12% (29:1) SDS-PAGE using 18 × 16-cm gels. The gels were silver stained (47), and bands were quantitated using densitometry. Binding curves were developed using the Hill equation to determine $K_{tr}$ and $n_H$ values with IGOR Pro software (WaveMetrics, Lake Oswego, OR). For cosedimentation, data from two separate batches of 36% and 38% glutathionylated G-actin were pooled.

**RESULTS**

**Changes in contractility with ischemia-reperfusion injury.** An in vivo rat model was used to determine the effect of ischemia-reperfusion injury on $F_{\text{max}}$, fiber stiffness, $K_{fr}$, $V_{\text{max}}$, and the force-$Ca^{2+}$ relationship of permeabilized cardiac muscle fibers (Fig. 1). Initially, the $Ca^{2+}$-activated $F_{\text{max}}$ of permeabilized trabeculae from control perfused, ischemic, or ischemia-reperfused hearts were compared in the presence of 5 mM ATP and 25 mM CP. The $Ca^{2+}$-activated $F_{\text{max}}$ produced was significantly reduced from 199 ± 32 mN/mm² ($n = 8$) in perfused fibers to 57 ± 8 mN/mm² ($n = 5$) ($P < 0.05$) in ischemic fibers. The $F_{\text{max}}$ of reperfused fibers (127 ± 16 mN/mm²; $n = 13$) was significantly higher than that of ischemic fibers ($P < 0.05$), although there was not full recovery to the $F_{\text{max}}$ levels generated by perfused fibers. The $EC_{50}$ increased significantly from 2.40 ± 0.11 μM [$Ca^{2+}$] in perfused fibers to 3.31 ± 0.28 μM [$Ca^{2+}$] in ischemic fibers ($P < 0.05$) but recovered with reperfusion to 1.86 ± 0.14 μM [$Ca^{2+}$].

The cooperativity of force activation (i.e., Hill coefficient) for perfused fibers was 6.28 ± 1.21, which was not significantly different from that in ischemic fibers (5.44 ± 0.47) or ischemia-reperfused fibers (5.38 ± 1.01). Fiber stiffness also declined significantly from 2.16 ± 0.28 N/mm² in perfused fibers to 1.44 ± 0.17 N/mm² in ischemic fibers ($P < 0.05$) but recovered in reperfused fibers to 2.18 ± 0.26 N/mm² ($P < 0.05$ vs. ischemic fibers). This decline in steady-state force and stiffness suggests that $K_{fr}$ might also be impaired with ischemia. Fibers were maximally $Ca^{2+}$-activated in the presence of 5 mM ATP and 25 mM CP, and then $K_{fr}$ values were measured. Similarly to the $F_{\text{max}}$ data, fibers from ischemic hearts showed the largest decline in $K_{fr}$, demonstrating a 44% drop from 12.43 ± 0.28 s⁻¹ in perfused fibers to 6.96 ± 0.38 s⁻¹ in ischemic fibers. The observed decrease in $K_{fr}$ with ischemia was largely recovered with reperfusion (11.00 ± 0.32 s⁻¹; $P < 0.05$ vs. ischemic fibers). To complement the measures of permeabilized fiber contraction under external load, $V_{\text{max}}$ was determined to provide a contrasting measure in the absence of tension redevelopment. In contrast to the $F_{\text{max}}$ and $K_{fr}$ results, $V_{\text{max}}$ values were not statistically different from control, ischemic, or ischemia-reperfused heart fibers (Table 1).

**Effect of ischemia-reperfusion injury on thin-filament proteins.** The contraction measurements in permeabilized fibers in the rat model suggest an effect of ischemia-reperfusion injury on the actomyosin filaments that resulted in impaired force generation against an external load. To explain these observations, previous studies demonstrated that ischemia-reperfusion injury of the heart may be accompanied by cleavage of thin-filament proteins. Specifically, TnI degradation was observed in models of ischemia-reperfusion injury and was postulated to

**Table 1. Contraction parameters from perfused, ischemic, and ischemia-reperfusion injury fibers**

<table>
<thead>
<tr>
<th>Data</th>
<th>Perfused</th>
<th>Ischemic</th>
<th>Ischemia-Reperfusion Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{\text{max}}$, mN/mm²</td>
<td>199±32</td>
<td>57±8*</td>
<td>127±16*</td>
</tr>
<tr>
<td>$Ca^{2+}_{50}$ μM</td>
<td>2.40±0.11</td>
<td>3.31±0.28*</td>
<td>1.86±0.14*</td>
</tr>
<tr>
<td>$n_H$</td>
<td>6.28±1.21</td>
<td>5.44±0.47</td>
<td>5.38±1.01</td>
</tr>
<tr>
<td>Stiffness, N/mm²</td>
<td>2.16±0.28</td>
<td>1.44±0.17*</td>
<td>2.18±0.26</td>
</tr>
<tr>
<td>$K_{fr}$, s⁻¹</td>
<td>12.43±0.28</td>
<td>6.96±0.38*</td>
<td>11.00±0.32</td>
</tr>
<tr>
<td>$V_{\text{max}}$, ML/s</td>
<td>2.65±0.30</td>
<td>3.02±0.40</td>
<td>2.65±0.24</td>
</tr>
</tbody>
</table>

Results are presented as averages ± SE from a minimum of 5 fibers. $F_{\text{max}}$, maximum force per cross-sectional area; $n_H$, Hill coefficient; $K_{fr}$, rate of tension redevelopment; $V_{\text{max}}$, maximum velocity of shortening. *$P < 0.05$ vs. perfused.
be a mechanism contributing to the depressed contractility of the myocardium (41, 54). In addition, recent data have suggested that TnT also may be a target of proteolysis (12, 13, 41). To examine these possibilities, left ventricular homogenates from perfused, ischemic, and ischemia-reperfused hearts were immunoblotted for cardiac TnI and TnT. Western blot analysis showed that the TnI 88-106 antibody did not detect degraded TnI (Figs. 2A and 2B). Examination of TnT in the left ventricular homogenates revealed an abundant low M, isoform corresponding to the predominant adult isoform as well as a much less abundant, higher M, isoform (29). However, no proteolytic fragments of TnT were observed. These results suggest that appreciable degradation of the thin-filament proteins TnI and TnT did not occur during the ischemia or reperfusion conditions tested.

In contrast to protein degradation, pathophysiological conditions that increase intracellular oxidative stress may result in covalent modification of proteins in situ. Previous studies have demonstrated that actin may be glutathionylated at COOH-terminal Cys374 as a result of oxidative stress (19, 20, 55, 56). Because glutathionylation has been suggested to be a modulator of protein activity (44, 55, 56), the possible glutathionylation of actin during ischemia was examined. Total profiles of perfused and ischemic heart homogenates were resolved by performing SDS-PAGE in the absence of an alkylator and immunoblotted using an anti-glutathione antibody (Fig. 3A). Both constitutively glutathionylated and selectively glutathionylated bands were observed under these conditions. Notably, a protein that migrated consistently with actin was observed to be glutathionylated in total ischemic extracts but was largely absent from total perfused extracts (Fig. 3A). This signal, although absent in the perfused sample, could also be abolished in the ischemic sample by treatment with DTT (Fig. 3B). In ischemic heart homogenates, an additional acidic actin isoelectric variant was observed on Western blot following two-dimensional SDS-PAGE, consistent with glutathionylation (Fig. 3C). This variant was abolished effectively after treatment with DTT, a sulfhydryl-reducing agent. Using densitometric quantitation, we found that the isoelectric point variant of actin accounted for 22 ± 6% (n = 4) of total actin in ischemic homogenates, reaching levels as high as 37% of total actin. To further ensure that this glutathionylated protein was actin, immunoprecipitation experiments were performed. Using homogenates from permeabilized ischemic left ventricles, the anti-actin antibody was used to immunoprecipitate actin under actin-depolymerizing conditions. The immunoprecipitated protein was then resolved by performing SDS-PAGE and subjected to Western blot analysis with either anti-actin or anti-glutathione antibody (Fig. 3D). The results demonstrate that the immunoprecipitated actin was also glutathionylated, confirming the nature of the posttranslational modification of actin. Furthermore, the use of extracts from permeabilized trabeculae suggests that the immunoprecipitated actin was derived largely from F-actin and therefore represents glutathionylated actin monomers that were integrated into the thin filaments. Nonetheless, additional experiments are required to absolutely exclude the possible contribution of nonfilamentous, free G-actin to the presented results.

**Effect of glutathionylation on actin polymerization.** To examine the effect of glutathionylation on actin’s function, purified G-actin was glutathionylated in vitro at Cys374 according to established protocols (16, 18). The in vitro glutathionylation of G-actin was verified using Western blot analysis with the anti-actin antibody (Fig. 3A). Additionally, glutathionylated G-actin was subjected to in vitro polymerization under conditions that absolutely exclude nonfilamentous actin. Immunoprecipitates were subjected to Western blot analysis with either anti-actin or anti-glutathione antibody (Fig. 3B). The results demonstrate that the immunoprecipitated actin was also glutathionylated, confirming the nature of the posttranslational modification of actin. Furthermore, the use of extracts from permeabilized trabeculae suggests that the immunoprecipitated actin was derived largely from F-actin and therefore represents glutathionylated actin monomers that were integrated into the thin filaments. Nonetheless, additional experiments are required to absolutely exclude the possible contribution of nonfilamentous, free G-actin to the presented results.
anti-glutathione antibody (Fig. 4). Furthermore, only one additional isoelectric variant of G-actin was evident on the basis of two-dimensional SDS-PAGE, consistent with glutathionylation at a single site (Fig. 4) (16). Quantitation of the actin isoelectric variants resolved by performing two-dimensional SDS-PAGE demonstrated that 30 ± 8% of the actin (n = 3) was glutathionylated, which is within the maximum level observed in vivo. Using F-actin light scattering, we determined the effect of G-actin glutathionylation on actin polymerization at three concentrations of total (glutathionylated + native) G-actin monomer. Pooled data from preparations of 17%, 36%, and 38% glutathionylated actin were compared with native G-actin preparations. Although the times for half-maximal actin polymerization were dependent on the starting monomer concentration (30) for both native and glutathionylated G-actin, glutathionylated G-actin polymerized slower than native G-actin at all concentrations tested (Fig. 5A). As a reference point, the time required for half-maximal polymerization of 10 μM native G-actin was 91 ± 3 s (n = 5), compared with 231 ± 21 s (n = 3) (P < 0.05) for glutathionylated G-actin. The maximum extent of polymerization was also reduced for glutathionylated G-actin compared with native G-actin (data not shown), consistent with prior work (55). In an additional set of polymerization experiments, a 10 μM solution of 38% glutathionylated G-actin was mixed with varying proportions of 10 μM native G-actin to reduce the relative amount of glutathionylated G-actin monomer while keeping the total monomer concentration constant. As the proportion of glutathionylated G-actin was reduced, the times for half-maximal polymerization were reduced in concert (Fig. 5B). This finding suggests that the effect of glutathionylated G-actin on actin polymerization kinetics was proportional across the range of glutathionylated monomer tested.

**Effect of actin glutathionylation on the interaction with Tm.** To further examine the impact of actin glutathionylation, its effect on the allosteric actin-Tm interaction was investigated (64). Actin polymerization experiments were conducted using either native or 38% glutathionylated G-actin in the presence of varying Tm concentrations. When mixed with 10 μM native G-actin, increasing concentrations of Tm dimer reduced the time required for half-maximal polymerization (Fig. 6A). However, at all Tm concentrations tested, the time course of polymerization for glutathionylated G-actin lagged that of native G-actin (Fig. 6B). The affinity of Tm for glutathionylated actin was determined by performing F-actin cosedimentation experiments using 2 μM native or glutathionylated F-actin and varying concentrations of Tm dimers (Fig. 7). Data from two batches of 36% and 38% glutathionylated F-actin were pooled. As determined using Hill fits of the data, *K*<sub>app</sub> for Tm binding to native F-actin was 3.4 ± 1.2 × 10<sup>6</sup> M<sup>−1</sup> (n = 4) vs. 2.5 ± 0.9 × 10<sup>6</sup> M<sup>−1</sup> (n = 4) for glutathionylated F-actin. The difference in these binding constants was not statistically significant. The Hill coefficient defining the cooperativity of the interaction between Tm and native F-actin was 2.84 ± 0.55 (n = 4), in contrast to 1.53 ± 0.28 (n = 4) for the interaction between Tm and glutathionylated F-actin. This difference in the cooperativity of Tm binding to F-actin was statistically significant (P < 0.05).

![Fig. 4. In vitro glutathionylation of globular actin (G-actin). Purified G-actin glutathionylated in vitro and examined using Western blot analysis with anti-glutathione antibody is shown at left. After glutathionylation, the anti-glutathione antibody identified the in vitro modified G-actin, and this signal was abolished by DTT treatment. To determine the extent of glutathionylation, an aliquot of glutathionylated G-actin was resolved by performing two-dimensional PAGE and silver stained. An aliquot of G-actin that was 38% glutathionylated is shown at right.](http://www.ajpcell.org/)

![Fig. 5. Effect of glutathionylation on actin polymerization. Polymerization of native or glutathionylated actin was studied using light scattering. A: using 5, 7.5, or 10 μM total monomer, native or glutathionylated G-actin was polymerized by adding KCl to 100 mM and MgCl<sub>2</sub> to 2 mM concentration. At all concentrations tested, polymerization of glutathionylated G-actin lagged that of native G-actin. B: to determine the effect of the extent of glutathionylation on actin polymerization, a 10 μM 38% glutathionylated G-actin sample was mixed with varying proportions of 10 μM native G-actin.](http://www.ajpcell.org/)
DISCUSSION

In the present study, we used an in vivo rat model of ischemia-reperfusion injury to examine force generation by permeabilized trabeculae after Ca^{2+} activation. The novel findings derived using this model stem from the observation that force generation by ischemic myocardium was depressed independent of ATP availability and that this deficit was largely recovered during reperfusion. These findings suggest that ischemia triggered intrinsic changes in the contractile filaments that altered their ability to generate force despite ATP availability. To better understand this decline in force generation by the permeabilized fibers, various contractile parameters, such as F_{\text{max}}, K_{\text{tr}}, V_{\text{max}}, and fiber stiffness, were measured and complemented with data on the force-Ca^{2+} relationships of the fibers. We have demonstrated that measures of fiber contraction under external load were affected adversely by ischemia but were largely recovered by reperfusion. Specifically, the decline in F_{\text{max}} and fiber stiffness associated with ischemia suggest that these alterations to the contractile apparatus adversely affect the activation of thin-filament regulatory units, resulting in a decline in attached cross bridges and, consequently, force (Table 1). However, we also note that despite the full recovery of fiber stiffness upon reperfusion from the ischemic state, the recovery of F_{\text{max}} was not complete. This divergence suggests that the intrinsic changes to the contractile filaments with ischemia may work to shift attached cross bridges to low- or non-force-producing states, rationalizing the remaining F_{\text{max}} deficit during reperfusion despite the full recovery of fiber stiffness. Future experiments focusing on ADP or P_{i}-dependent transitions of the cross-bridge cycle may better describe the cause of the divergence in force and fiber stiffness during reperfusion.

The reversible decline in force generation of the permeabilized fibers also suggests that proteins of the contractile filaments may be altered during ischemia-reperfusion injury. Previous research demonstrated that proteolytic degradation of the thin-filament proteins TnI and TnT may contribute to myocardial stunning (12, 41, 54). Proteolysis would be consistent with a hypothesized change to the actomyosin filaments that could alter contractility independent of ATP availability. However, the decline in force generation with ischemia was largely recovered by reperfusion, indicating that proteolysis would need to be recovered rapidly by protein synthesis and reintegration within 60 min of reperfusion. Although this consideration indirectly suggests that proteolysis was not a major contributor to the decline in force generation observed in this model, it was confirmed using Western blot analysis for TnI and TnT, two subunits of the troponin complex that were previously observed to be truncated in alternate models (Fig. 2).

Rather than proteolysis of thin-filament proteins, the data present suggest that alternate modifications to proteins of the contractile apparatus may be responsible for the observed changes. Recent studies have highlighted the role of post-translational modifications, such as phosphorylation or acetylation, in modulating contractility. These modifications can alter the affinity and cooperativity of cross-bridge interactions, thereby affecting force generation independently of ATP availability. Future investigations focusing on these modifications may provide insights into the mechanisms underlying the observed divergences in force and fiber stiffness during reperfusion.
actomyosin filaments occurred with ischemia. Prior research established that actin may be glutathionylated in response to cellular oxidative stress, resulting in the modification of the physiologically labile COOH-terminal Cys residue (55). In the present study, examination of ischemic heart homogenates demonstrated that actin was glutathionylated in our in vivo model (Fig. 3), suggesting that actin may be a novel contributor to the observed change in force generation with ischemia. Because we analyzed proteins from permeabilized fibers in the present study, these findings suggest that the glutathionylated actin was myofibrillar rather than monomeric G-actin in a soluble pool. The impact of actin glutathionylation was tested in vitro using purified G-actin that had been glutathionylated at the COOH-terminal Cys (Fig. 4). The time course of polymerization for glutathionylated G-actin lagged that of native G-actin at all concentrations tested (Fig. 5A), in agreement with previous observations (16, 55). This decrease in polymerization was proportional to the percentage of glutathionylated G-actin monomer present, suggesting that the impact of this covalent modification on actin's function depended on the fraction of actin in the thin filament that was glutathionylated in situ during ischemia-reperfusion injury (Fig. 5B). In prior work, it was demonstrated that in vitro modification of this COOH-terminal end of actin, through either glutathionylation or amino acid removal, destabilized actin filaments as well as supporting actin filament reorganization (17, 40, 51, 56). It was therefore postulated that the deficit in polymerization of glutathionylated actin could be rescued by stabilizing the actin-actin contacts with Tm. Although Tm decreased the time required for half-maximal polymerization in all cases, the time course of polymerization for glutathionylated G-actin remained slower than that for native G-actin (Fig. 6). Therefore, the data reported in the present study suggest that glutathionylation of actin within the observed physiological range impaired actin intermonomer contacts.

These findings were further investigated by determining the affinity of Tm for native and glutathionylated F-actin. Tm’s inability to rescue the polymerization activity of glutathionylated actin may have been interpreted as a decrease in Tm affinity for glutathionylated actin. However, cosedimentation experiments did not reveal a significant difference in Tm affinity for native vs. glutathionylated F-actin. Rather, a statistically significant decline in the cooperativity of Tm binding to glutathionylated F-actin was observed (Fig. 7). Although there are no data supporting direct contact of Tm with Cys374 of actin (37), the cosedimentation results suggest that the allosteric interaction between Tm and actin was affected by actin glutathionylation. This finding is consistent with the results of prior work suggesting that cooperative binding of Tm to F-actin involved conformational changes propagated along the actin filament subsequent to Tm binding (8, 25, 38, 59). These allosteric changes in actin after Tm binding are supported by cross-linking experiments demonstrating that contact between myosin S1 and actin residues 48–67 could be abrogated by the presence of Tm (3), despite the absence of direct contact between Tm and actin at these residues (36). In addition, cleavage between residues 42 and 43 of the DNase I binding loop of actin attenuated the inhibitory effect of Tm on actin-activated myosin S1 ATPase activity (38), suggesting that the DNase I binding loop is sensitive to conformational changes subsequent to Tm binding. These data complement data regarding the intermonomer interactions of actin indicating that the DNase I binding loop and the COOH-terminal Cys374 of the adjacent actin are sufficiently close in proximity to be cross linked efficiently by an ~12-Å linker (26). Specifically, Gln41 of actin was cross-linked to Cys374 of the laterally adjacent actin, indicative of intermonomer contacts between actins. These results suggest that in situ glutathionylation of actin in the thin filament during ischemia-reperfusion injury may alter the contractile performance of the myocardium. Glutathionylation may modify actin intermonomer interactions that take place subsequent to Tm dimers shifting with thin-filament activation. Alternatively, the noted shift of Tm during muscle activation may be impaired by the change in actin intermonomer contacts due to glutathionylation. This cross talk between actin and Tm is consistent with observations that cooperative Tm binding to actin is dependent on a degree of propagation through the actin filament (25) and builds on the observation that Tm binding to actin produces a conformational change within the actin filament (8). Additional experiments are necessary to develop a better understanding of the impact of altered actin-Tm interactions on force generation. Along these lines, previous work examining a Tm mutation associated with human cardiomyopathies demonstrated a weakened interaction between the mutant Tm and actin in vitro (23). Nonetheless, the impact of this mutation in fibers was observed as a change in the Ca2+ sensitivity of force generation (4). Because the Tm-actin interaction itself is not Ca2+ sensitive, it is not entirely clear how changes in the interaction of these two proteins predicts the observed effect of sensitizing fibers to Ca2+. In light of these data, it is not clear whether the change in the cooperativity of association observed in the present study (Fig. 7) must translate specifically to a decrease in the cooperativity of force activation, although a small decline in nH was observed (Table 1). However, glutathionylated actin’s possible contribution to Ca2+ desensitization during ischemia cannot be ruled out. Future studies detailing the effect of actin glutathionylation will require a methodical characterization of its interaction with other individual thin-filament proteins, as well as its effect on the regulated in vitro actomyosin ATPase. These studies will lead to more comprehensive models to describe the allosteric interactions that regulate force generation by the contractile filaments.

Additional experiments are also warranted to examine the multifocal effect of ischemia and ischemia-reperfusion injury on cross-bridge activation in muscle fibers. A large body of evidence suggests that beyond ATP availability, posttranslational modifications such as protein oxidation or phosphorylation (52, 53) of contractile filament proteins also underlie the observed net change in force generation by the myocardium, especially for acute conditions in which ventricular remodeling cannot be a factor. Therefore, future experiments will require comprehensive in vitro systems to examine the impact of unique posttranslational modifications effectively, not only by themselves but also in combination. Such an integrated approach will better define the deleterious and protective modifications whose net impact defines the function of the contractile apparatus during and after ischemia-reperfusion injury.

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