Specific enhancement of sarcomeric response to Ca\textsuperscript{2+} protects murine myocardium against ischemia-reperfusion dysfunction

Grace M. Arteaga,\textsuperscript{1,2,3} Chad M. Warren,\textsuperscript{1,3} Sanja Milutinovic,\textsuperscript{2,3} Anne F. Martin,\textsuperscript{1} and R. John Solaro\textsuperscript{1,3}

\textsuperscript{1}Center for Cardiovascular Research, \textsuperscript{2}Department of Pediatrics, and \textsuperscript{3}Department of Physiology and Biophysics, College of Medicine, University of Illinois at Chicago, Chicago, Illinois

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Arteaga, Grace M., Chad M. Warren, Sanja Milutinovic, Anne F. Martin, and R. John Solaro. Specific enhancement of sarcomeric response to Ca\textsuperscript{2+} protects murine myocardium against ischemia-reperfusion dysfunction. Am J Physiol Heart Circ Physiol 289: H2183–H2192, 2005. First published July 15, 2005; doi:10.1152/ajpheart.00520.2005.—Alteration in myofilament response to Ca\textsuperscript{2+} is a major mechanism for depressed cardiac function after ischemia-reperfusion (I/R) dysfunction. We tested the hypothesis that hearts with increased myofilament response to Ca\textsuperscript{2+} are less susceptible to I/R. In one approach, we studied transgenic (TG) mice with a constitutive increase in myofilament Ca\textsuperscript{2+} sensitivity in which the adult form of cardiac troponin I (cTnI) is stoichiometrically replaced with the embryonic/neonatal isoform, slow skeletal TnI (ssTnI). We also studied mouse hearts with EMD-57033, which acts specifically to enhance myofilament response to Ca\textsuperscript{2+}. We subjected isolated, perfused hearts to an I/R protocol consisting of 25 min of no-flow ischemia followed by 30 min of reperfusion. After I/R, developed pressure and rates of pressure change were significantly depressed and end-diastolic pressure was significantly elevated in nontransgenic (NTG) control hearts. These changes were significantly blunted in TG hearts and in NTG hearts perfused with EMD-57033 during reperfusion, with function returning to nearly baseline levels. Ca\textsuperscript{2+}- and cross bridge-dependent activation, protein breakdown, and phosphorylation in detergent-extracted fiber bundles were also investigated. After I/R NTG fiber bundles exhibited a significant depression of cross bridge-dependent activation and Ca\textsuperscript{2+}-activated tension and length dependence of activation that were not evident in TG preparations. Only NTG hearts demonstrated a significant increase in cTnI phosphorylation. Our results support the hypothesis that specific increases in myofilament Ca\textsuperscript{2+} sensitivity are able to diminish the effect of I/R on cardiac function.

troponin I; calcium sensitizers; phosphorylation; stunning; cross bridge-dependent activation

MYOCARDIAL INJURY AND STUNNING resulting from ischemia and subsequent reperfusion (I/R) involve complex effects on regulation and structure of the myocardium. Among these effects is a depressed contractile function of the myocardium associated with a prominent decrease in myofilaments to Ca\textsuperscript{2+} (16, 19). One proposed molecular mechanism for this effect has been proteolysis of sarcomeric proteins, especially cardiac troponin I (cTnI) (49). I/R-induced proteolytic cleavage of 18 amino acids at the COOH terminus of TnI has been demonstrated to depress maximal tension similar to that seen in stunning (34). Moreover, transgenic (TG) mice expressing this truncated form of cTnI exhibit stunning. Although the role of this proteolytic cleavage of cTnI appears to be more prominent in rats than in other species, there is general agreement that disturbance of the integrity of myofilament proteins such as proteolysis and oxidation is a major contributing factor to I/R (27). There is also the possibility that posttranslational modifications of sarcomeric proteins, which depress maximum myofilament tension, are associated with I/R-induced activation of kinases such as PKC (44) and the mitogen-activated protein kinase p38 MAP kinase (26).

In experiments reported here, we have tested whether hearts with increased myofilament sensitivity to Ca\textsuperscript{2+} demonstrate a resistance to injury after I/R. We used a TG mouse model expressing slow skeletal troponin I (ssTnI) in place of cTnI (10). This model demonstrates a constitutive increase in myofilament response to Ca\textsuperscript{2+} (2) and has the additional advantage that ssTnI is not a substrate for PKA or PKC (48). There is stoichiometric isoform switching of cTnI with ssTnI in this model, with no evidence of histological or structural changes in the hearts and with little or no effect on Ca\textsuperscript{2+} fluxes (10). We also induced an increase in myofilament response to Ca\textsuperscript{2+} with the use of the Ca\textsuperscript{2+}-sensitizing agent EMD-57033. EMD-57033, a thiadiazinone derivative, promotes the actin-myosin interaction (39) by a mechanism that is independent of phosphodiesterase inhibition and that may involve docking at the COOH-terminal region of cardiac troponin C (cTnC) (46). An important rationale of our experiments is based on emerging evidence of the potential importance of stimulating sarcomeric activity as a therapy for heart failure (1, 24) and stunning (31). Although few Ca\textsuperscript{2+} sensitizers have been tested in clinical trials, currently levosimendan (Simdax) has proven to be a strong candidate for use in the management of low-output heart failure and ischemic left ventricular failure management in humans (13, 31). It is apparent that levosimendan binds to the NH\textsubscript{2}-terminal regulatory region of cTnC and thereby increases tension at a given level of Ca\textsuperscript{2+} (40). Earlier clinical experience with the Ca\textsuperscript{2+} sensitizer pimobendan (Acardi) has also indicated the potential therapeutic advantages of this class of cardiotonic agents (15).

Whether the beneficial effects of these Ca\textsuperscript{2+} sensitizers are due to an increased myofilament response to Ca\textsuperscript{2+} remains an open question. An important issue with regard to the use of levosimendan and pimobendan is that both agents have been demonstrated to have effects other than direct activation of the sarcomere. Both agents demonstrate inhibition of phosphodiesterase (8, 12). Moreover, levosimendan also acts to open K\textsuperscript{+} channels (51), and pimobendan has effects on cytokines (7, 37). Thus our TG mouse model expressing ssTnI in the cardiac compartment is ideal to test whether specific enhancement of

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sarcomeric response to Ca\(^{2+}\) is able to blunt the deleterious effects of I/R. Our data demonstrate that specifically increasing Ca\(^{2+}\) sensitivity either by the use of EMD-57033 or by transgenesis improves cardiac and myofilament function after I/R. This improvement appears to occur independently of proteolytic damage to the myofilament proteins. Our results have important implications with regard to current therapeutic management for cardiac conditions associated with sustained or transient depressions in contractility.

**MATERIALS AND METHODS**

All investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, Revised 1985) and were approved by the Institutional Animal Review Board of the University of Illinois at Chicago.

**ssTnI TG Mice**

The generation and characterization of the TG mice used in this report were described in detail previously (10).

**Heart Isolation and Perfusion**

A total of 40 CD strain female mice, weighing between 25 and 30 g, were studied. The mice were deeply anesthetized by intraperitoneal injection with pentobarbital sodium (25 mg/kg) after being anticoagulated for 20 min with heparin (100 U/Ip). A median sternotomy was performed, and the heart was rapidly excised and placed in cold Krebs-Henseleit (K-H) buffer (described below). After dissection and visualization, the ascending aorta was carefully cannulated under microscopic guidance with a stainless steel 22-gauge newborn feeding needle 25 mm in length with a ball diameter of 1.25 mm (Popper & Sons, New Hyde Park, NY). To avoid air embolization of the coronaries, we filled the cannula in advance with K-H solution by attaching its proximal end to a 1-ml syringe containing the buffer. The aorta was secured on the cannula with a 4-0 silk suture before transfer to the perfusion apparatus. During transfer, the cannula was intermittently perfused with K-H solution under low pressure to prevent air entry. Once the cannula was attached to the perfusion apparatus, retrograde perfusion at constant pressure (60 mmHg) was initiated. The time from the excision of the heart to the commencement of perfusion was recorded and always maintained at <2.5 min as recommended by Sumery and Yellon (42).

**Perfusion Buffer and Pressure Determination**

All chemicals used for the K-H solution were obtained from Fisher Scientific (Fair Lawn, NJ). The hearts were perfused with a modified K-H solution containing (in mM) 118.0 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 25.0 NaHCO\(_3\), 1.2 NaH\(_2\)PO\(_4\), 10.0 glucose, and 0.5 EDTA. The solution was saturated with a mixture of 95% O\(_2\) and 5% CO\(_2\) (pH 7.4), and the temperature was continuously maintained at 37°C. A small incision at the pulmonary outflow tract was made to allow free drainage of coronary effluent. For positioning of a latex balloon in the left ventricle, the left atrium was excised at its base and the balloon was inserted through the mitral valve. Left ventricular diastolic pressure was set between 5 and 10 mmHg with the use of a microsyringe. The perfused mouse hearts were placed inside a glass water-jacketed chamber and electrically stimulated at a rate of 360 beats/min with copper electrodes attached to the base and apex of the heart. The stimulation was performed with pulses of alternating polarity and 1.5-ms duration, with the voltage set at 50% above the threshold level. The contractile function of these isolated hearts was measured with a 1.4-Fr Millar pressure catheter (model SPR-671, Millar Instruments, Houston, TX) positioned inside a closed water column system connected to the latex balloon at the distal end. The proximal end of the catheter was attached to a pressure transducer control unit (model TCB-500, Millar Instruments) and connected to a control unit, Power Lab/4sp (AD Instruments, Castle Hill, Australia), allowing transduced left ventricular pressure and heart rate to be monitored continuously during the experiment. From the left ventricular developed pressure (LVEDP), we calculated positive (+dP/dt) and negative (−dP/dt) derivatives with Chart for Windows 5.01 software (AD Instruments).

**Ventricular Balloon Preparation**

The latex balloons were prepared from stretched small pieces of thin plastic bags 0.012 mm thick (0.55 mil) without tearing the film. A 20-gauge stainless hypodermic needle was cut in half, and one end was affixed to a 10-cm piece of PE-90 tubing while the other end secured the plastic film with a 4-0 suture material to form a small balloon as described by Hampton et al. (17). An appropriate balloon was considered to have a capacity of 35–40 μl. A 4-0 silk thread 5 cm in length affixed to a small, blunted sewing needle was carefully secured at the distal end of the balloon to allow for easy introduction into the left ventricle. The developed pressure was evaluated automatically from the signal with an online computer.

**Experimental Protocol**

During each experiment, the heart was maintained inside a water jacket at constant temperature of 37°C. After 25–30 min of stabilization, baseline values for developed pressure were recorded. We excluded from our study any hearts in which coronary flow rate was excessive (>5 ml/min, indicating a possible aortic tear) or demonstrated spontaneous bradycardia (<300 beats/min), poor contractility (<60 mmHg), or persistent arrhythmia after 20 min of stabilization. TG and nontransgenic (NTG) hearts were then exposed to 25 min of no-flow global ischemia. Temperature was carefully controlled at 37°C. The ischemic period was followed by 30 min of reperfusion (Fig. 1), and recovery of pressure and heart rate were continuously recorded. Pacing of the heart was maintained for 3 min after initiation of ischemia and then stopped completely. Pacing was restarted 3 min after the initiation of reperfusion. Developed pressure was measured in both groups throughout the experimental procedure. The values were expressed as a percentage of baseline developed pressure. After hearts completed a period of 30 min of reperfusion, the balloon was deflated and retracted and both ventricles were flash frozen in liquid nitrogen and saved for further biochemical determinations. In experiments using the Ca\(^{2+}\) sensitizer, we perfused the hearts with K-H buffer containing 3 μM EMD-57033 only during the reperfusion phase. We used 3 μM EMD-57033 based on previous work (2) and on a pilot dose-response experiment in which 50% of maximal developed force in skinned fiber preparations was obtained at pCa 6 (data not shown).

**Gel Electrophoresis**

**Sample preparation.** Approximately 20–30 mg of liquid nitrogen-frozen mouse left ventricle was homogenized in a Dounce homogenizer in 1 ml of sample buffer [8 M urea, 2 M thiourea, 0.05 M Tris-HCl (pH 6.8), 75 mM DTT. 3% SDS, 0.05% bromphenol blue] (50). Myofibrils were prepared for the determination of phosphorylation status from 20–30 mg of mouse left ventricle by homogenization in 1 ml of relaxing buffer (in mM: 75 KCl, 10 imidazole pH 7.2, 2 MgCl\(_2\), 2 EGTA, 10 EDTA, 1 Na\(_2\)HPO\(_4\), 4 phosphocreatine, 1 ATP, 50 2,3-butanediol monoamine, 1 DTT, and 1 benzamidine-HCl) and 1% (vol/vol) Triton X-100. The pellet was then homogenized three more times in rigor buffer (in mM: 75 KCl, 10 imidazole pH 7.2, 2 MgCl\(_2\), 2 EGTA, 10 EDTA, and 1 mM Na\(_2\)HPO\(_4\)) with 1% (vol/vol) Triton X-100. The pellets were then washed twice with rigor buffer to remove the Triton X-100. The pellets were finally washed twice with...
buffer containing (in mM) 60 KCl, 20 MOPS pH 7.0, and 2 MgCl₂ and solubilized in sample buffer. All buffers used for the myofibrillar preparation had protease and phosphatase inhibitors (0.25 mM PMSF, 1.25 mg/ml leupeptin, 1.25 mg/ml pepstatin, 50 mM NaF, and 0.25 mM Na₃VO₄). The protein concentrations of the samples were determined with a RC-DC assay kit (Bio-Rad, Hercules, CA). The samples were loaded onto the gels at 50 μg/lane and 7 μg total protein/lane for proteolysis and phosphorylation determination, respectively.

Gel electrophoresis and Western blotting. SDS-PAGE was performed in a Criterion gel system (Bio-Rad). The resolving gel was 15% total acrylamide and cross-linked with 0.5% bisacrylamide pH 8.8, and the stacking gel was 2.95% total acrylamide and cross-linked with 15% total acrylamide and cross-linked with 0.5% bisacrylamide pH 6.0 formed in a Criterion gel system (Bio-Rad). The resolving gel was loaded onto the gels at 50 μg/lane and 7 μg total protein/lane for proteolysis and phosphorylation determination, respectively.

To detect phosphoproteins specifically, the gel was stained with Pro-Q Diamond gel stain (Molecular Probes, Eugene, OR). The gel was fixed in 120 ml of 10% trichloroacetic acid-50% methanol for 1 h, with two changes followed by washout in 120 ml of water for 10 min two times, and placed into 80 ml of Pro-Q Diamond gel stain for 1.5 h. Finally, the gel was destained in 15% 1,2-propanediol, 50 mM sodium acetate pH 4.0 for 45 min. Fresh destaining solution was added, and the gel was allowed to shake overnight. On the next day, the gel was imaged with an FX Pro Plus imager (Bio-Rad) in conjunction with a CY3 filter set. The gel was subsequently stained with 20 ml of SYPRO Ruby for 1 h and destained with 10% methanol, 10% acetic acid for at least 30 min to visualize total protein. The phosphorylated proteins detected with Pro-Q Diamond stain were normalized to actin stained with SYPRO Ruby to equalize minor loading discrepancies. The quantitative optical densities of the bands from both Pro-Q Diamond- and SYPRO Ruby-stained gels were determined with Imagequant version 5.2 (Amersham Biosciences). The optical densities were imported into Microsoft Excel, and the ratio of phosphorylated protein to actin was determined and used to compare relative phosphorylation levels.

Skinned Fiber Preparations and Force Measurements

We measured force developed by bundles of detergent-extracted fibers dissected from papillary muscle as previously described (2). Hearts were excised as described above and placed in cold high-relaxing (HR) buffer (in mM): 10 EGTA, 2 free Mg²⁺, 5 MgATP²⁻, 79.2 KCl, 5.4 Na₂ATP, 12 creatine phosphate, and 20 MOPS, pH 7.0 (ionic strength 150 mM) with 2.5 μg/ml pepstatin A, 1 μg/ml leupeptin, and 50 μM PMSF. Papillary muscle was isolated, and bundles of fibers (~150 μm in diameter and 4–5 mm long) were prepared. The fibers were mounted between a force transducer and a movable post with fast-setting glue and immersed in HR buffer that contained 10 IU/ml creatine kinase. Sarcomere lengths were set at 1.9 or 2.3 μm with laser diffraction. Isometric pCa-tension relations were determined by banishing the skinned fiber bundles at 22°C sequentially in low-relaxing buffer, in 0.1 mM EGTA in place of the 10 mM EGTA in HR, and then in solutions of various Ca²⁺ concentrations ([Ca²⁺]) generated by adding CaCl₂ to HR as computed with a
computer tension was plotted as a function of $[Ca^{2+}]$ and fitted to the Hill equation by applying nonlinear least-squares regression analysis with Prism software (GraphPad version 2.0). Isometric tensions measured at submaximally activating $[Ca^{2+}]$ were expressed as a percentage of the maximum tension. Half-maximally activating $[Ca^{2+}]$ were determined from individual Hill fits of each $[Ca^{2+}]$-tension relation and then averaged.

Force-MgATP relationships were determined at 22°C in a $Ca^{2+}$-free solution (pCa 9) containing (in mM) 20 MOPS (pH 7.0), 1 free Mg$^{2+}$, 10 EGTA, and 12 creatine phosphate with 10$^{-8}$-10$^{-3}$ M MgATP$^{2-}$. Ionic strength was adjusted to 150 mM with KCl, and free $Ca^{2+}$ was set at 10$^{-9}$ M. The midpoint and Hill coefficient of the force-pMgATP relationships were determined by fitting data to the Hill equation as described for the pCa-force relations.

Statistical Analysis

Data from isolated hearts and skinned fiber bundles are expressed as means ± SE. Differences in the time course of contractile function after sustained ischemia and during reperfusion were statistically evaluated by a two-way analysis of variance among the groups. In the skinned fiber bundle preparations, data in which measurements were made in the same fiber bundle at different sarcomere lengths were analyzed individually with Student’s paired $t$-test. When three or more groups were analyzed, a one-way repeated-measures ANOVA was used with a Newman-Keuls multiple-comparison test. The results were taken to be significant when $P < 0.05$.

RESULTS

Myocardial Function During Perfusion Alone and After I/R

To investigate the role of myofilament $Ca^{2+}$ sensitivity in promoting tolerance to I/R, we used a well-characterized TG model (TG-ssTnI) that expresses 100% of ssTnI isoform in adult mouse heart instead of cTnI. To further determine the effect of increased $Ca^{2+}$ sensitivity in murine myofilaments exposed to I/R, we used a well-known $Ca^{2+}$-sensitizing agent with no phosphodiesterase activity, EMD-57033 (1). Langendorff preparations were used to induce a cardiac global no-flow ischemia for a period of 25 min followed by a 30-min reperfusion. Figure 1A shows the LVDP in the I/R protocol in groups of NTG, TG-ssTnI, and NTG hearts exposed to EMD-57033 at a 3 μM concentration (NTG EMD) during reperfusion. Compared with NTG controls, there was a statistically significant enhancement in left ventricular function after I/R in hearts of mice expressing the ssTnI isoform and in hearts reperfused with the $Ca^{2+}$ sensitizer EMD-57033. These results indicate that mechanisms targeting the thin filament and modulating $Ca^{2+}$ sensitivity reverse the myocardial dysfunction associated with I/R. Figure 1B shows the changes in enddiastolic pressure (EDP) occurring in the three studied groups during reperfusion. The increase in EDP, evident in the NTG hearts during the reperfusion phase (contracture), did not return to baseline conditions after 30 min of reperfusion. However, EDP in the TG ssTnI and NTG EMD groups returned to levels close to those present before ischemia. Figure 1C reports the changes in $+\Delta p/dt$ and Fig. 1D the changes in $-\Delta p/dt$ that occurred during I/R. Under baseline conditions, $+\Delta p/dt$ and $-\Delta p/dt$ were not significantly different in the TG-ssTnI and NTG hearts. However, after 30 min of reperfusion, $+\Delta p/dt$ fell to nearly 50% of baseline values, whereas $+\Delta p/dt$ of TG hearts and NTG EMD hearts returned to baseline levels after I/R. In NTG hearts, $-\Delta p/dt$ fell to nearly 40% of the baseline value.

However after I/R, in NTG EMD hearts, $-\Delta p/dt$ was nearly the same as baseline. In TG-ssTnI hearts, $-\Delta p/dt$ rose above baseline.

pCa-Tension Relations in Skinned Fiber Preparations

Data summarized in Fig. 2 show the findings from experiments with skinned fiber preparations obtained from NTG and TG-ssTnI hearts perfused for 50 min and hearts from both groups that had been subjected to the I/R protocol. Table 1 summarizes the data and demonstrates important differences between the NTG and TG-ssTnI fiber bundles. Figure 2A shows the pCa-tension relations of NTG fiber bundles perfused only and after I/R. I/R induced a significant decrease in maximum tension and a decrease in myofilament $Ca^{2+}$ sensitivity. In comparison, a significant finding is presented in Fig. 2B, where the TG-ssTnI fiber bundles maintained force after I/R. Maximal tension fell substantially in the NTG controls but was unaffected in the TG-ssTnI fiber bundles. This finding remained significant when fibers from the NTG and TG-ssTnI...
groups after I/R were compared (Table 1). As previously reported (2), compared with NTG controls, TG-ssTnl fiber bundles were more sensitive to Ca\(^{2+}\) and less sensitive to a change in sarcomere length (Table 1). These characteristics remained in fiber bundle preparations isolated from hearts after 50 min of perfusion alone.

Table 1 compares the length dependence of activation at two sarcomere lengths, 1.9 \(\mu\)m and 2.3 \(\mu\)m, both in the NTG preparations perfused and after I/R and in the TG-ssTnl preparations perfused and after I/R. We found that under our conditions there was no change in length dependence of activation in the TG-ssTnl preparations, whereas there was a significant decrease in length dependence of activation in the NTG I/R group.

**Cross Bridge-Dependent Activation in Skinned Fiber Preparations**

The alteration in length dependence of activation associated with I/R indicates a potential alteration in the ability of strong cross bridges to activate the thin filament. We therefore determined cross bridge-dependent activation explicitly by incubating the skinned fiber bundles in a range of increasing MgATP concentrations at pCa 9.0. As illustrated in Fig. 3, as MgATP concentration is lowered, rigor complexes form that activate nucleotide containing cross bridges despite the absence of Ca\(^{2+}\). Skinned NTG fiber bundles isolated from hearts stressed by I/R showed a significant reduction in cross bridge-dependent activation, as illustrated by the leftward shift of the pMgATP-force relation (Fig. 3A). This decrease in cross bridge-dependent activation was less pronounced in the ssTnl-TG skinned fiber bundles (Fig. 3, B and D). In skinned fiber bundles from hearts subjected to perfusion alone without I/R, the TG-ssTnl preparations showed a reduced cross bridge-dependent activation compared with NTG control skinned fiber bundles isolated from perfused hearts stressed by I/R (Fig. 3C).

We also tested the direct effects of EMD-57033 in cross bridge-dependent activation of skinned fiber bundles isolated from NTG and TG-ssTnl hearts after perfusion alone or after I/R. Data in Fig. 4A show that EMD-57033 had no significant effect on fiber bundles isolated from NTG-perfused hearts. However, EMD-57033 did induce a sensitization to rigor cross bridge activation in preparations isolated from NTG hearts stressed by I/R. As shown in Fig. 4A, in the case of fiber bundles from TG-ssTnl hearts, EMD-57033 induced a significant sensitization to rigor cross bridges in preparations isolated from both perfused-alone and I/R hearts. Fig. 4, C and D,
compare data from skinned fiber bundles isolated from NTG and TG-ssTnI hearts perfused alone (Fig. 4C) or subjected to I/R (Fig. 4D).

**TnI Proteolysis and Myofilament Protein Phosphorylation After I/R**

The etiology of the myocardial dysfunction resulting from I/R has been attributed to the breakdown of the COOH-terminal region of cTnI (34). To investigate this hypothesis in our I/R model, we isolated myofibrillar preparations obtained from both NTG and TG-ssTnI hearts subjected to perfusion alone for 45 min or to I/R. Figure 5 shows data from NTG and TG-ssTnI hearts subjected to I/R or perfusion alone. Our results demonstrate that the NTG group did not show proteolysis of TnI under any of these conditions when probed with the C5 antibody (amino acids 186–192; data not shown). However, we detected a slight degradation of cTnI by using the M46 antibody targeting cTnI amino acids 130–145 (Fig. 5A). Figure 5B compares this degradation to the amount of ssTnI degradation in the perfused and I/R models. Although I/R did not induce an increase in cTnI proteolysis, there was a small but significant increase in ssTnI proteolysis in the TG hearts. We conclude from these data that the protection from I/R is not due to a lower extent of TnI proteolysis in ssTnI-TG hearts compared with NTG controls.

We also determined the role of myofibrillar protein phosphorylation as a potential mechanism for the differences in susceptibility to I/R in NTG and TG-ssTnI hearts. Figure 6 shows a SYPRO Ruby-stained gel for total protein (Fig. 6A) and a Pro-Q Diamond-stained gel specific for phosphorylated proteins (Fig. 6B). I/R did not induce a change in myosin light chain (MLC2) phosphorylation in either the NTG or TG-ssTnI hearts (Table 2). However, levels of MLC2 phosphorylation were higher in NTG hearts than in TG-ssTnI hearts, whether perfused only or subjected to I/R. There were no significant differences in phosphorylation of troponin T (TnT) and myosin binding protein C (MyBP-C) between NTG and TG-ssTnI hearts, whether perfused alone or stressed by I/R. I/R did, however, induce a significant ($P < 0.05$) increase in cTnI phosphorylation in the NTG hearts. As expected, we observed no phosphorylation of ssTnI under all experimental conditions.

**DISCUSSION**

Our results provide new insights into the mechanism of I/R and the potential that specific modifications at the level of the sarcomere may be an important therapeutic approach. Novel insights include the identification of a depressed length-dependent and cross bridge-dependent activation as an element in I/R. Moreover, our data provide a correlation between the...
extent of depression in maximum tension of myofilaments and recovery from I/R.

Differences between ssTnI and cTnI that reside in COOH-terminal regions are likely to be important in the relative protection of TG-ssTnI hearts against the dysfunction associated with I/R. Previous studies reported a clip of 17 COOH-terminal amino acids of cTnI and implicated modifications in the COOH-terminal region as causal in depressing maximum tension in I/R injury and stunning (34). In fact, hearts of TG mice expressing a small amount of the cTnI(1–193) mutant demonstrated stunning (34). However, in the studies reported here, we could not attribute the depression of maximum tension in the NTG skinned fiber bundles induced by I/R to a proteolysis of sarcomeric proteins. There was no breakdown of cardiac TnT (cTnT), and there was a similar and relatively small amount of proteolytic breakdown of TnI in both the NTG and TG hearts that occurred independently of I/R. Although we could not find increased generation of the truncated form of cTnI in our protocol, an important role of the COOH-terminal region of cTnI in I/R is likely. Previous studies from our laboratory (25) using skinned fiber bundles containing recombinant chimeras of fast skeletal TnI (fsTnI) and cTnI showed that differences in COOH-terminal regions of these isoforms could account largely for relative insensitivity of fast skeletal myofilament to deactivation by acidic pH. This region of cTnI differs from fsTnI and ssTnI in having an Ala residue at position 162 that corresponds to a His residue in the skeletal variants. Dargis et al. (5) reported that reconstituted preparations containing mutant cTnI(A162H) demonstrated a blunting of pH sensitivity of the Ca\textsuperscript{2+}-actomyosin ATPase rate relation similar to that of preparations containing fsTnI. The region of cTnI that contains Ala 162 is adjacent to the "switch peptide" that shuttles between binding to actin and to the NH\textsubscript{2} lobe of cTnC depending on whether Ca\textsuperscript{2+} is bound to the single regulatory site (43). Thus this region is critical to sarcomeric inhibition and release from inhibition by Ca\textsuperscript{2+}. It is likely that this regional difference between ssTnI and cTnI is also respon-

Table 2. Myofilament protein phosphorylation

<table>
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<tr>
<th>Protein</th>
<th>NTG Perf</th>
<th>TG Perf</th>
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<th>TG I/R</th>
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<tr>
<td>MLC2</td>
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<td>0.13±0.06</td>
<td>0.25±0.02†</td>
<td>0.17±0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE ratios of phosphorylated protein to actin as described in MATERIALS AND METHODS. MyBP-C, myosin binding protein C; TnI, troponin I; TnT, troponin T; MLC2, myosin light chain 2. *P ≤ 0.05 between NTG Perf and NTG I/R; †P ≤ 0.05 between NTG I/R and TG, both Perf and I/R.
sible in part not only for protection against I/R and deactivation by acidic pH in TG-ssTnI hearts but also for our recently reported finding (23) of significant protection of TG-ssTnI hearts against cardiac dysfunction induced by endotoxemia. It is also apparent that differences in PKA- and PKC-induced deactivation of the NTG and TG-ssTnI myofilaments, which lack the NH2-terminal extension, may also be important in protection against endotoxemia.

An important distinction between myofilaments from TG and NTG hearts is our finding of a significant effect of I/R on cross bridge-dependent activation of the preparations containing cTnI, with no effect of I/R on the preparations containing ssTnI. This difference may be a significant factor in the lack of effect of I/R on maximum tension on the TG skinned fiber bundles compared with the controls, in which maximum tension fell by 30% after I/R. There is substantial evidence that full activation of the thin filament requires both Ca2+ and strong, force-generating cross bridges (28, 29). There is also evidence that specific changes in TnI isoform populations and isoform-specific interactions between TnI and cTnC affect the ability of strong cross bridges to activate the thin filament. Morimoto et al. (33) reported that when cTnC was extracted from skinned fiber bundles, a drop in pH no longer was able to depress cross bridge-dependent activation. The inability of acidic pH to affect cross bridge-dependent activation in preparations missing cTnC suggests that key signaling pathways for cross bridge-dependent activation involve the COOH lobe of cTnC and an NH2-terminal region of cTnI comprised amino acids 33–80. Under the conditions of the experiment (pCa 9.0) carried out by Morimoto et al. (33), this interface forms the tightest interaction between cTnC and the rest of the thin filament. Moreover, when ssTnI replaced cTnI in the skinned fiber bundles, the ability of acidic pH to depress cross bridge-dependent activation was significantly blunted. Thus we think that, apart from differences between COOH-terminal regions of cTnI and ssTnI described above, differences in the NH2-terminal regions of ssTnI and cTnI, which form the interface with the COOH lobe of cTnC, are important elements in protection of TG-ssTnI hearts from I/R. This idea fits with our finding that EMD-57033, which protected against I/R, also protected against TG-ssTnI. Finley et al. (11) reported that binding of cTnI to a phosphormimetic form of cTnI with Asp residues replacing Ser43/Ser45 induced destabilization of helix G in the COOH lobe of cTnC. Thus, the ability of EMD-57033 to protect against I/R may be due in part to its binding to the COOH lobe of cTnI and ability to overcome the negative effects of PKC-dependent phosphorylation on the cTnI-cTnC interaction.

Although one might expect that increased myofilament sensitivity to Ca2+ would increase energy demand and diastolic abnormalities, which would further exacerbate the detrimental effects of I/R, we found that TG hearts with constitutive increases in myofilament response to Ca2+ recovered better than controls. A possible explanation for this beneficial effect of increased myofilament Ca2+ sensitivity is a lowering of ATP demand for cellular (especially sarcoplasmic reticulum) Ca2+ fluxes. Another possible explanation is conservation of ATP hydrolysis by increased economy of contraction in the myofilaments of TG-ssTnI hearts compared with NTG controls. Recent data from our laboratory (45) showed that maximum Ca2+-activated actomyosin ATPase rate of TG-ssTnI myofilaments determined in loaded skinned fiber bundles is ~20% lower than that of NTG controls. Importantly, TG-ssTnI myofilaments demonstrate a significant decrease in tension cost (unit of ATPase activity/unit of tension generated). This decrease in tension cost is similar to that which occurs with increases in the relative proportion of β-myosin heavy chain in the isoform population (35) of myosin heavy chain, which also protect against I/R (3). Blunt et al. (3) also correlated an increase in postischemic function with a depression in actomyosin ATPase rate in hearts expressing the mutant form of cTnT(F1101). The presence of the F1101 mutation in cTnT also sensitized the myofilaments to Ca2+, which fits our interpretation that protection of postischemic function may involve increased Ca2+ sensitivity as well as increased economy of tension development.

A striking finding in our experiments was the improvement of diastolic pressure in the TG-ssTnI and NTG EMD hearts versus controls after I/R. This finding is important with regard to the use of Ca2+-sensitizing agents as therapeutic tools in reperfusion of the ischemic myocardium, and these results
emphasize the importance of understanding the mechanisms by which an increase in myofilament Ca\(^{2+}\) sensitivity improves the response of the heart to stressors. For example, levosimendan, which sensitizes myofilaments to Ca\(^{2+}\), has also been demonstrated to increase cardiac function with no change in the balance between ATP generation and consumption (9). The clinical experience with the administration of levosimendan is that of improved recovery after cardiopulmonary bypass (40). Furthermore, the use of levosimendan in patients with severe, low-output heart failure resulted in improved hemodynamics compared with dobutamine, and mortality was decreased after 6 mo (13). Understanding the mechanisms underlying the beneficial effects of increased myofilament sensitivity to Ca\(^{2+}\) in I/R expands our armamentarium of approaches to medical management of patients with acquired or congenital cardiac disease, as well as with stunned myocardium after cardiopulmonary bypass.

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