Absence of Troponin I Degradation or Altered Sarcoplasmic Reticulum Uptake Protein Expression After Reversible Ischemia in Swine

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Abstract—The findings of troponin I (TnI) proteolysis (in isolated rat hearts) and induction of selected sarcoplasmic reticulum (SR) calcium-regulatory genes (after repetitive total coronary occlusions in swine) have given rise to the hypothesis that the time course of functional recovery of stunned myocardium reflects the resynthesis of reversibly damaged proteins. Although stunning occurs after brief total occlusions and prolonged partial occlusions (ie, short-term hibernation), the time course of functional recovery varies from a few hours to several days, suggesting that the severity of protein damage or mechanisms responsible for the dysfunction may differ. To study this, we examined SR gene expression and TnI degradation in stunned myocardium produced by 10-minute total left anterior descending coronary artery (LAD) occlusions (n=4) or 1-hour partial LAD occlusions, in which flow was reduced to ≈50% of control values for 60 minutes (n=6) in swine. One hour after reperfusion, LAD wall thickening was severely depressed in both models despite normal perfusion and no triphenyltetrazolium chloride evidence of necrosis. Normal myocardium exhibited TnI immunoreactivity at 31 kDa and a weak secondary band at 27 kDa. Irreversible injury or calpain activation in vitro produced a marked increase in the intensity of the 27-kDa band, consistent with TnI degradation. Stunned myocardium demonstrated no change in the 31- or the 27-kDa band, and the percentage of the 27- to 31-kDa band remained constant after 10-minute total occlusions (LAD, 5.9±0.9%; normal, 4.9±1.6%) and 1-hour partial occlusions (LAD, 8.5±1.9%; normal, 7.3±1.4%) and in sham controls (LAD, 10.9±1.5%; normal, 9.8±1.4%). Northern analysis showed no alterations in TnI or SR gene expression, but the stress protein HSP-70 was variably induced. Thus, stunned myocardium occurs without TnI degradation or altered SR gene expression, indicating that additional mechanisms are responsible for the reversible dysfunction after single episodes of regional ischemia in swine. **(*Circ Res*. 1999;85:446-456.)**

Key Words: ischemia ■ myocardial stunning ■ troponin I ■ sarcoplasmic reticulum ■ HSP-70

Transient occlusion of a coronary artery (or low-flow ischemia) is accompanied by a rapid reduction in contractile function. After 20 minutes, glycogen is largely depleted and reductions in ATP reach critical levels, at which irreversible sarcolemmal injury and myocyte necrosis begin in vivo.[1] Reperfusion before 15 minutes in vivo is associated with persistent contractile dysfunction in the absence of necrosis that has been termed myocardial stunning.[2] Although the precise mechanisms of stunning continue to be unclear, the time course of recovery is consistent with the notion that it may involve the resynthesis of proteins involved in contractile function that are damaged after reperfusion. This is presumably effected by feedback control mechanisms activating transcription of their respective genes to increase mRNA levels and resynthesize damaged proteins back to control levels. In support of this hypothesis are recent studies demonstrating troponin I (TnI) proteolysis in stunned myocardium after 15 to 20 minutes of global ischemia in isolated rat hearts[3,4] and increased mRNA levels for a variety of proteins after 2 sequential 10-minute total coronary occlusions in swine.[5] Other laboratories have, however, reported negative findings with regard to TnI proteolysis, suggesting that it may only occur with concomitant irreversible injury.[6,7] These divergent results may arise from methodological differences, species variations, in vitro versus in vivo conditions, and/or the lack of a standard assessment of myocardial viability in most studies to exclude confounding tissue necrosis. It is also possible that the variability of findings and the importance of protein degradation may be due to the severity of the ischemic insult. For example, stunning after single 10-minute occlusions resolves in 6 hours,

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whereas stunning after a 5-hour partial occlusion (ie, short-term hibernation model) can last up to 7 days.

We performed the present study to determine whether stunned myocardium after a period of short-term hibernation from a 1-hour partial coronary occlusion altered sarcoplasmic reticulum (SR) gene expression and TnI degradation in a fashion similar to that of stunning from a brief total occlusion in swine. Alterations of protein and mRNA that might be due to an admixture of reversibly and irreversibly injured tissue were excluded by using occlusion durations that are associated with completely reversible injury and confirmed by triphenyltetrazolium chloride (TTC) staining in each experiment. The expression of selected SR genes and TnI was assessed by Northern blotting with porcine-specific cDNAs. TnI proteolysis was assessed using Western analysis with 2 TnI antibodies. The results contrast with findings in other models of stunned myocardium and demonstrate that dysfunctional myocardium after single episodes of reversible ischemia in pigs is not accompanied by regional alterations in TnI levels, TnI proteolysis, or early alterations in SR gene expression. Positive control experiments demonstrate that TnI degradation in swine is only seen in association with irreversible myocyte injury.

Materials and Methods

All experimental procedures and protocols conformed to institutional guidelines for the care and use of animals in research.

One-Hour Partial Coronary Occlusion Model of Stunning

Acutely Instrumented Animals (n=10)

Pigs (56.9±5.2 kg) were premedicated with a Telazol (50 mg/mL tiletamine and 50 mg/mL zolazepam)/xylazine [100 mg/mL] mixture (0.037 mL/kg IM), intubated, and mechanically ventilated. A surgical plane of anesthesia was maintained with α-chloralose (60 mg/mL; initial bolus 80 mg/kg followed by 20 to 30 $mg \cdot kg^{-1} \cdot h^{-1}$; n=9) or halothane (1 to 2%, n=1) throughout the duration of the experiment. Arterial blood gases were regularly monitored via a catheter in the femoral artery, and body temperature was maintained with a heating pad.

The heart was exposed through a midline sternotomy, and the left anterior descending coronary artery (LAD) was dissected free from its surrounding tissue. A hydraulic occluder was placed around the artery, and a Doppler flow probe was positioned proximal to the occluder for monitoring coronary flow velocity. Left ventricular (LV) pressure was measured with a Millar catheter or a high-fidelity micromanometer (Konigsberg, model P6.5) inserted into the apex. Catheters were placed in the aorta and left atrium. Regional wall thickening was obtained using piezoelectric crystals. One crystal of each pair was inserted tangentially into the subendocardium, and the other was attached to a polyethylene terephthalate (Dacron) patch sewn onto the epicardial surface. Crystal pairs were placed in the anterior and posterior freewall, and subendocardial positioning was confirmed at the end of the study.

Chronically Instrumented Animals (n=11)

A second group of pigs (45.0±2.1 kg) was chronically instrumented before experimental protocols. Pigs were fasted overnight and premedicated with a Telazol/xylazine mixture (0.037 mL/kg IM). They were intubated and mechanically ventilated, and a surgical plane of anesthesia was maintained with an isoflurane (1 to 3%)/oxygen mixture. The heart was exposed through a left thoracotomy in the fourth or fifth interspace. Each animal was instrumented as described for the first group, with the exception that wall thickening was measured with a single epicardial crystal system (Crystal Biotech, Inc). The chest was closed, and 2% lidocaine (injected to produce an intercostal nerve block) and butorphanol (0.025 mg/kg IM) were administered for analgesia. Prophylactic antibiotics (gentamicin, 60 mg IM; cephalothin, 500 mg IV) were given before surgery and repeated immediately after closing the chest. Studies were conducted in the closed-chest state after allowing the animals to recover for at least 1 week. Six of the animals were subjected to ischemia, and 5 served as sham controls. During the experimental study, the animals were intubated and mechanically ventilated, and anesthesia was induced and maintained as described above for instrumentation.

Experimental Protocol

After a 30-minute stabilization period, ischemia was induced by partially inflating the hydraulic occluder until LAD wall thickening approached akinesis. This was maintained for 60 minutes, after which the occluder was released, followed by a 60-minute reperfusion period. Regional myocardial blood flow was assessed by injecting colored microspheres suspended in saline with thimerosal (0.01%) and Tween 80 (0.01%) immediately before the partial occlusion, at the end of the 60-minute ischemic period, and 1 hour after releasing the occlusion. Microsphere suspensions were sonicated and vortex agitated before injection. Approximately 3 million microspheres (15 μm in diameter), labeled with 1 of up to 4 different-colored dyes (yellow, red, white, and blue), were administered as a bolus via the left atrial catheter and flushed with saline. An arterial reference withdrawal sample was started before microsphere injection and continued for 90 seconds at a rate of 6.0 mL/min. At the end of the reperfusion period, AC fibrillation or KCl injection euthanized the pig. The heart was quickly excised, and transmural blocks of tissue were cut from core areas of the area perfused by the LAD (ischemic region) and the normally perfused region. Each block was further subdivided into subendocardial, midmyocardial, and subepicardial samples. Microsphere perfusion was analyzed by eluting the color dyes from tissue samples as previously described.[8] Samples for RNA and protein analyses were flash frozen in liquid nitrogen and stored at −80°C until analyzed. Myocardial necrosis was evaluated by TTC staining.

Brief Total Occlusion Model of Stunning (n=6)

Six pigs were sedated with Telazol/xylazine (0.037 mL/kg IM), intubated, and anesthetized with isoflurane (1% to 3%; balance, oxygen). The heart was exposed via a sternotomy and a hydraulic occluder placed around the mid-LAD. Pairs of ultrasonic crystals were placed to measure LAD wall thickening. Regional flow was assessed with microspheres injected through a LV catheter and sampled through a carotid catheter. After equilibrating for 20 minutes, resting flow and hemodynamics were assessed, and the animals were given prophylactic lidocaine (1 mg/kg followed by 0.5 mg/kg 5 minutes later and an additional 1 mg/kg 5 minutes before release of the occlusion). The LAD was totally occluded for 10 minutes, and microsphere flow and hemodynamics were repeated at the end of the occlusion period. After release of the occlusion, the hearts were reperfused for 1 hour, after which flow and hemodynamic measurements were repeated and the hearts excised for sampling as outlined above. Two animals developed ventricular fibrillation on reperfusion and were excluded, because they did not complete the protocol.

Global Ischemia in Isolated Rat Hearts (n=6)

In an additional series of experiments, we quantified the ability of the C5 monoclonal TnI antibody to detect TnI degradation after global ischemia in a constant flow Langendorff preparation using methodology identical to that previously described by Gao et al.[3] Briefly, rats (LBN-F1 strain, 200 to 250 g, Harlan Sprague Dawley, Inc, Indianapolis, Ind) were anesthetized with sodium pentobarbital (60 mg/kg IP). The hearts were exposed through a sternotomy and, after heparinization (3000 units into the left atrium), rapidly excised. The aorta was cannulated and perfused retrogradely (15 mL/min) with Krebs-Henseleit solution equilibrated with 95% oxygen/5% CO_2 having the following electrolyte composition (in mmol/L): NaCl

TABLE 1. Hemodynamics, Flow, and Function: Acutely Instrumented 1-Hour Partial Occlusion (n=10)

	Preischemic Control	1-Hour Partial Occlusion	1-Hour Reperfusion
HR, bpm	100±4	100±5	100±5
P_{A0} systolic, mm Hg	103±5	94±4	88±4
P_{A0} mean, mm Hg	88±5	79±5	74±4
LAD wall thickening, percentage	22.3±3.8	−0.1±1.4*	6.1±2.2*
LV dP/dt, mm Hg/s	1833±139	1376±90*	1312±105*
LAD Endo flow, mL · min^{-1} · g^{-1}	0.96±0.10	0.31±0.05*	0.86±0.09

Values are mean±SEM. HR indicates heart rate; P_{A0}, aortic pressure; LV dP/dt, first derivative of LV pressure; and Endo, subendocardial.

*$P<0.05$ vs Rest.

120, $NaHCO_3$ 20, $MgCl_2$ 1.2, glucose 10, and $CaCl_2$ 1.0 at a pH of 7.35 to 7.40. The hearts were paced at 275 bpm throughout the experiments, with the exception that pacing was turned off during ischemia and resumed during reperfusion. Isovolumic LV pressure was measured with a water-filled latex balloon (Radnotti, Inc) that was connected to a pressure transducer (model CDX3, Cobe). The heart was immersed in a water-filled container, and temperature was measured by a thermistor in the right ventricle and maintained at 37°C. Initial diastolic LV filling pressure was adjusted to a nominal value of 10 mm Hg, except in one of the sham control hearts that was studied at an elevated preload.

Hearts were divided into the following groups: (1) 2 hearts rapidly excised for control TnI measurements, (2) 2 hearts that underwent 20 minutes of control buffer perfusion followed by 20 minutes of global ischemia and reperfusion for 20 minutes, and (3) 2 hearts that served as sham controls to assess the effects of buffer perfusion at a normal (10 mm Hg) and elevated (20 mm Hg) preload for 60 minutes. Left ventricles were rapidly frozen and processed for Western analysis as described below.

RNA Isolation and Northern Analysis

Total myocardial RNA was isolated from flash-frozen samples by guanidinium thiocyanate/phenol-chloroform extraction, vacuum blotted to a nylon membrane (Zeta-Probe GT, Bio-Rad), and fixed with ultraviolet light. Hybridization with selected cDNA probes labeled with [α-^{32}P]dCTP was performed overnight at 65°C.

The following pairs of oligonucleotide primers were used to amplify probes from porcine cDNA, with the exception of HSP-70 and the SR Ca^{2+} ATPase, for which the human and rat cDNA were used, respectively: (1) HSP-70,[9] sense primer, 5′-GATCCTAGCAAATGACCAGG-3′ (+81 to +100 nt), and antisense primer, 5′-TTGAGCCCTGCAATGGCACC-3′ (+503 to +484 nt), 423-bp product; (2) SR Ca^{2+} ATPase,[10] sense primer, 5′-TTGGCTTGGTTCGAAGAAGG-3′ (+223 to +242 nt), and antisense primer, 5′-CCAAGAGCCACCATGAACTG-3′ (+864 to +845 nt), 642-bp product; (3) calsequestrin,[11] sense primer, 5′-AAGCTTGCCAAGAAGCTGGG-3′ (+301 to +320 nt), and antisense primer, 5′-GCAAAGGCCACAATGTGGAT-3′ (+821 to +802 nt), 521-bp product; (4) phospholamban,[12] sense primer, 5′-TCAGCTTTCTCTTGACGGCT-3′ (−52 to −33 nt), and antisense primer, 5′-ACCCCTAGTTCATCCTCAGA-3′ (+474 to +455 nt), 526-bp product; (5) TnI,[13] sense primer, 5′-GGAGAGAAGGGGCGCGCTCTGAG-3′ (+297 to +319 nt), and antisense primer, 5′-CCTCCCGGTTTTCCTTCTCGGTGTC-3′ (+651 to +627 nt), 355-bp product; and (6) GAPDH,[14] sense primer, 5′-TTCACCACCATGGAGAAGGC-3′ (+300 to +319 nt), and antisense primer, 5′-TGTCATACCAGGAAATGAGC-3′ (+942 to +923 nt), 640-bp product. Probes were labeled by PCR in the presence of [α-^{32}P]dCTP (30 cycles at 94°C, 30 seconds; 50°C, 1 minute; and 72°C, 2 minutes). The hybridization signals (beta emissions) were quantified on a PhosphorImager (Molecular Dynamics, Inc). To control for small variations in RNA loading, the total volume of each band was divided by the total volume of its corresponding GAPDH signal. Only the high molecular mass band of phospholamban was tabulated.

Protein Isolation and Immunoblotting for TnI

Protein was isolated from flash-frozen subendocardial samples using an extraction buffer containing (in mmol/L) Tris (pH 7.4) 20, NaCl 50, EDTA (pH 8) 1, β-mercaptoethanol 5, sodium vanadate 0.2, and phenylmethylsulfonyl fluoride 0.2, as well as 1 μg/mL pepstatin, 0.5 μg/mL leupeptin, and 10% SDS. Total protein (10 to 50 μg/lane) was electrophoresed and separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell), which was soaked in 3% nonfat dry milk in PBS. Membranes were rinsed in PBS and incubated overnight with a goat polyclonal antibody to human TnI (Biodesign International) at a dilution of 1:4000. Subsequently, membranes were rinsed in PBS and incubated with horseradish peroxidase (HRP)–protein G (Zymed Laboratories) diluted in PBS (1:5000) for 1 hour. Bands were visualized using a HRP developer, and background-subtracted signals were quantified on a laser densitometer (Bio-Rad). Linearity of density and protein loading was demonstrated over a range of 2 to 50 μg. We also repeated TnI quantification with a second monoclonal antibody specific for bovine TnI (clone C5, Biogenesis). Gels were loaded at 200 μg/lane, transferred to Immobilon-P membranes (Millipore, Inc), and incubated overnight at an antibody dilution of

TABLE 2. Hemodynamics and Function: 10-Minute Total Occlusion (n=4)

	Preischemic Control	Occlusion	Reperfusion
HR, bpm	80±8	82±10	81±7
P_{A0} systolic, mm Hg	112±6	109±5	118±4
P_{A0} mean, mm Hg	84±4	83±4	88±5
LAD wall thickening, percentage	13±3	−3±3*	1±2*
LV dP/dt, mm Hg/s	1203±135	1123±100	1082±122
LAD Endo flow, mL · min^{-1} · g^{-1}	0.67±0.05	0.09±0.02*	0.60±0.03

Values are mean±SEM. Abbreviations as in Table 1.

*$P<0.05$ vs Rest.

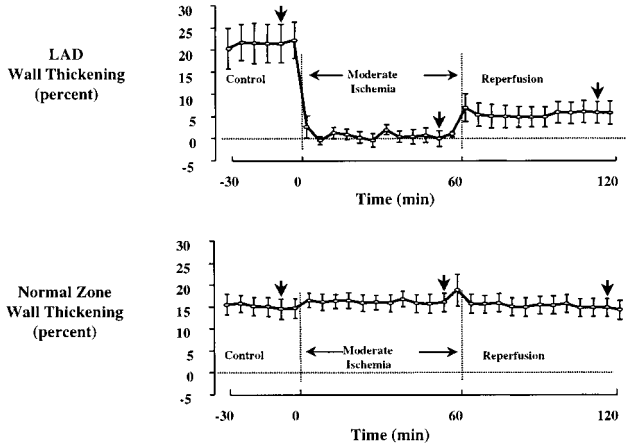

Figure 1. Temporal changes in LAD and normal zone wall thickening. LAD wall thickening (top) averaged 22.3±3.8% under control conditions and decreased to −0.1±4.1% ($P<0.05$ vs control) during ischemia. Wall thickening improved to 6.1±2.2% ($P<0.05$ vs control) after 1 hour of reperfusion, yet was still significantly reduced from baseline. Normal zone wall thickening (bottom) remained constant throughout the protocol. Arrows indicate time points at which microspheres were injected. Values are mean±SEM.

1:2000. They were rinsed in PBS, incubated with HRP–protein G (1:10 000) for 1 hour, and quantified as outlined above.

Data Analysis

Hemodynamic parameters were recorded on a Gould model 2800W recorder and digitized on a Gateway 2000 computer (sampling rate, 200 Hz) using the Dataflow Analysis System (Crystal Biotech, Inc). All data are expressed as mean±SEM except for the rat experiments, for which individual data are presented. Differences between the LAD and normal regions were compared using an ANOVA, and paired *t* tests were used for 2 sample means. Sham and experimental group comparisons were made using *t* tests assuming equal variances.

Results

All animals were in good health at the time of study, and TTC staining revealed no myocardial necrosis after a 1-hour partial or 10-minute total occlusion in swine.

Effects of Reversible Ischemia on SR Calcium-Handling Proteins

Hemodynamics, function, and subendocardial perfusion in open-chest anesthetized pigs are summarized in Tables 1 and 2. With the exception of LAD wall thickening and flow, hemodynamics remained unchanged during the course of the experiments. Figure 1 demonstrates the stability of wall thickening at 5-minute intervals in pigs subjected to a partial coronary occlusion for 1 hour. Under control conditions, LAD wall thickening averaged 22.3±3.8% and was not significantly different from values in the normally perfused region. A partial LAD occlusion resulting in akinesis (LAD wall thickening, −0.1±1.4%, $P<0.05$ versus control) reduced LAD flow from 1.02±0.11 to 0.45±0.06 mL · min^{-1} · g^{-1}. After release of the occlusion, flow returned to control values (0.88±0.09 mL · min^{-1} · g^{-1}), but wall thickening remained depressed (6.1±2.2%, $P<0.05$). Table 2 summarizes hemodynamics, flow, and function in pigs subjected to a single 10-minute total LAD occlusion followed by 1 hour of reperfusion. Wall thickening during occlusion was dyskinetic, and function remained severely depressed after 1 hour of reperfusion, despite the fact that flow returned to resting values. Thus, both prolonged partial occlusion and brief total occlusion models resulted in regionally stunned myocardium in the absence of irreversible myocardial injury.

TABLE 3. Summary of Phospholamban and SR Ca^{2+} ATPase mRNA Changes After Single Episodes of Reversible Ischemia

	Average mRNA/GAPDH (Densitometric Units)			
	Normal	LAD	*P*	n
1-hour partial occlusion				
Phospholamban	7.7±1.6	7.5±1.6	NS	10
SR Ca^{2+} ATPase	3.9±0.7	4.0±0.72	NS	10
Calsequestrin	2.5±0.13	2.4±0.12	NS	8
HSP-70	1.0±0.2	4.8±1.9	<0.05	16
10-minute total occlusion				
Phospholamban	1.4±0.3	1.3±0.1	NS	4
SR Ca^{2+} ATPase	6.7±0.4	6.6±0.7	NS	4
HSP-70	4.4±1.6	25.2±12.3	0.14	4

Values are mean±SEM. NS indicates no significant difference, LAD vs Normal.

The expression of selected SR proteins after a 1-hour partial coronary occlusion is summarized in Figure 2. There were no changes in mRNA levels for the SR Ca^{2+} ATPase, calsequestrin, or phospholamban. The expression of GAPDH, which was used to control for RNA loading, also remained unchanged and averaged 3.02±0.60 densitometric units in LAD regions and 3.01±0.64 densitometric units in corresponding normal regions (P=NS). Table 3 summarizes results for the expression of the SR Ca^{2+} ATPase, phospholamban, and HSP-70 in subendocardial samples obtained at 1-hour reperfusion after a partial or brief total coronary occlusion. There were no alterations in the SR Ca^{2+} ATPase

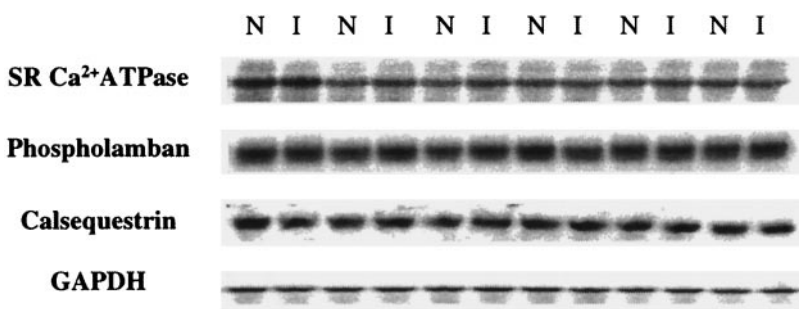

Figure 2. Subendocardial mRNA for the SR Ca^{2+} ATPase, phospholamban, calsequestrin, and GAPDH from normal (N) and previously ischemic (I) regions. There was no regional difference in mRNA for these candidate genes 1 hour after release of a partial coronary occlusion. Similar results were found after 10-minute total occlusions.

TABLE 4. Hemodynamics, Flow, and Function: Chronically Instrumented, 1-Hour Partial Occlusion

	Preischemic Control		1-Hour Partial Occlusion		1-Hour Reperfusion	
	Experimental	Sham	Experimental	Sham	Experimental	Sham
HR, bpm	90±3	93±5	86±4	92±8	88±6	86±3
P_{A0} systolic, mm Hg	120±6	114±6	113±9	117±5	104±8	104±8
P_{A0} mean, mm Hg	98±4	96±5	94±6	100±3	84±7	87±9
LAD wall thickening, percentage	17.0±3.1	25.0±2.5	3.3±0.9*†	19.7±0.7	4.4±2.5*†	21.2±1.4
LV dP/dt, mm Hg/s	1433±132	1326±94	1253±114	1276±109	1095±91	1063±110
LAD Endo flow, mL · min^{-1} · g^{-1}	1.29±0.16	1.41±0.08	0.41±0.06*†	1.34±0.18	1.11±0.09	1.34±0.31

Values are mean±SEM; n=6 partial occlusion and n=5 sham. Abbreviations as in Table 1.
*$P<0.05$ vs preischemic controls; †$P<0.05$ vs sham.

or phospholamban after a single episode of ischemia in either protocol. In contrast, HSP-70 in the dysfunctional LAD region was induced 5-fold.

Effects of Reversible Ischemia on TnI Degradation

Hemodynamics, function, and regional perfusion in closed-chest anesthetized animals subjected to a partial coronary occlusion and sham controls are summarized in Table 4. The hemodynamic changes, wall thickening, and coronary flow were similar to those summarized above. Myocardial perfusion averaged 1.24±0.13 mL · min^{-1} · g^{-1} under control conditions and fell to 0.55 mL · min^{-1} · g^{-1} at the end of the 1-hour partial occlusion. Wall thickening in the LAD region fell from 17.0±3.1% to 3.3±0.9% and remained depressed at 1 hour (4.4±2.5%, $P<0.05$) despite restoration of perfusion (1.02±0.07 mL · min^{-1} · g^{-1}, P=NS versus control and shams). There were no changes in hemodynamics or regional perfusion over time in sham-instrumented animals.

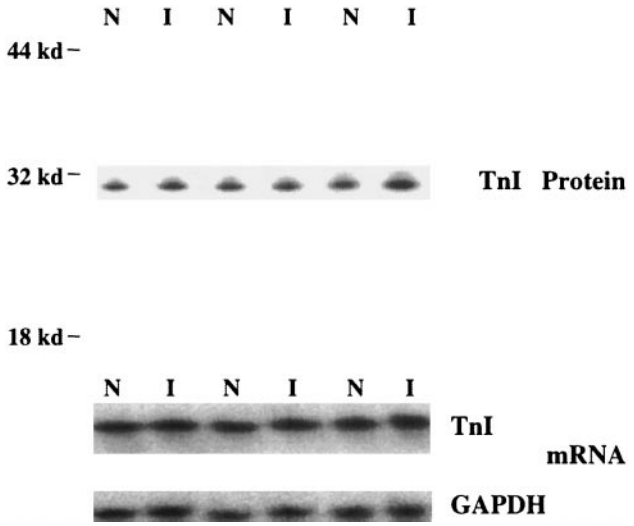

Figure 3. Western analysis of subendocardial tissue for TnI using the polyclonal TnI antibody (top) in chronically instrumented pigs subjected to a 1-hour partial occlusion followed by 1 hour of reperfusion. The lower gels show the corresponding mRNA for TnI and GAPDH by Northern analysis. Western analysis demonstrated a single band at ≈31 kDa when loaded at a concentration of 10 μg/lane. At higher loading concentrations, a variable-intensity lower molecular mass band was identified that was similar in normal (N) and reversibly ischemic (I) samples (Table 5). Northern analysis showed no alteration in mRNA levels for TnI or GAPDH.

Figure 3 summarizes the effects of ischemia on TnI mRNA and protein levels (polyclonal antibody) in paired samples from 3 representative animals. There was no change in the mRNA levels of TnI 1 hour after release of a partial occlusion (LAD 4.3±0.3 densitometric units and 4.5±0.2 densitometric units in normal). Similarly, a single 10-minute total occlusion did not alter TnI mRNA levels (LAD 2.8±0.1 versus 2.9±0.1 in normal). Western analysis using the polyclonal TnI antibody demonstrated a primary band at 31 kDa that was similar in LAD and normal regions (Figure 3). There was a faint secondary band at a molecular mass of 27 kDa (0 to 2.4% of the 31-kDa band). The appearance of this band varied with the amount of protein loaded, but, as summarized in Table 5, it was not altered in stunned myocardium after a partial or total coronary occlusion.

Because TnI antibodies may vary in their ability to detect the lower molecular mass band, we repeated the immunoblots using the C5 monoclonal antibody and performed 3 additional positive control experiments to confirm its ability to detect TnI degradation in the pig. The first positive control (Figure 4, inset) shows an increase in the TnI degradation band in irreversibly injured LAD myocardium from a porcine heart in which a myocardial infarct had been produced 24 hours earlier. The TnI degradation band increased from 10% in the normal region to 31% in infarcted myocardium. The graph summarizes the relation between optical density and protein loading for the C5 monoclonal antibody. There was a linear increase in the optical density of the 27-kDa band with total protein concentration over a range of 10 to 600 μg/lane. In contrast, the 31-kDa TnI band optical density increased nonlinearly with protein concentration, particularly as loading exceeded 60 μg/lane (similar to the results for the polyclonal antibody). In the subsequent experiments, we overloaded gels with 200 μg/lane to ensure that we could reliably detect and quantify even small changes in the 27-kDa band.

In a second positive control experiment, we produced irreversibly injured tissue by incubating porcine myocardium at 37°C in vitro using methodology similar to that previously described by others in dogs and rats.[15,16] A Western blot summarizing results for the C5 antibody is illustrated in Figure 5. There was a small increase in the degradation band after 1 to 1.5 hours followed by a marked increase at 24 hours with the appearance of multiple low molecular mass TnI degradation bands. In dogs, the onset of irreversible injury in

TABLE 5. Densitometric Analysis of TnI Immunoblots After Reversible Ischemia

		31 kDa, Densitometric Units		27 kDa, Densitometric Units		27 kDa/31 kDa, %	
	n	NI	LAD	NI	LAD	NI	LAD
Polyclonal TnI antibody							
1-hour partial occlusion	6	5.2±0.3	5.8±0.3	0.1±0.1	0.2±0.1	1.4±1.0	2.4±1.2
10-minute total occlusion	5	9.2±0.5	8.7±0.5	0	0	0	0
Monoclonal TnI antibody							
1-hour partial occlusion	6	14.7±0.5	14.4±0.9	1.08±0.2	1.15±0.2	7.3±1.4	8.5±1.9
10-minute total occlusion	4	14.3±1.4	12.6±2.7	0.64±0.2	0.69±0.1	4.9±1.6	5.9±0.9
Sham	5	11.4±1.9	11.0±1.8	1.1±0.2	1.3±0.4	9.8±1.4	10.9±1.5

Values are mean±SEM. For the polyclonal antibody results, gels were loaded with 20 μg/lane and 50 μg/lane for partial and total occlusion experiments, respectively. The gels with the monoclonal antibody were loaded at 200 μg/lane in all groups.

this model begins at ≈1.5 hours and is coincident with the increase in the 27-kDa TnI band in swine myocardium.[15]

Finally, in a third positive control experiment, we incubated protein preparations for 60 minutes with Ca^{2+} to activate endogenous proteases and demonstrate that the low molecular mass band is consistent with calpain-mediated TnI proteolysis in the pig. Figure 6 shows that there was a weak 27-kDa band under control conditions that increased in the presence of 10 mmol/L Ca^{2+}. The increase in the 27-kDa band could be blocked by incubating the tissue with EDTA (0.1 mol/L) to bind Ca^{2+} or by blocking calpain with the addition of calpeptin (10 μg). An increase in the lower 27-kDa band could also be demonstrated with the polyclonal antibody (data not shown). Thus, these positive control experiments demonstrate that a 27-kDa band can be detected in normal myocardium that increases substantially in swine after interventions associated with irreversible myocardial injury in vitro and in vivo.

Figure 7 summarizes results for the C5 TnI antibody in pigs with stunned myocardium after a prolonged partial or brief total occlusion. As in the experiments described above, porcine myocardium demonstrated a weak 27-kDa band under normal conditions that did not change in stunned myocardium 1 hour after reperfusion. Densitometric findings are summarized in Table 5, and paired results from each animal (27-kDa band expressed as a percentage of TnI) are summarized for a 1-hour partial occlusion and a 10-minute total occlusion and for sham pigs in Figure 8. Neither the TnI band nor the degradation band changed in any of the experimental groups. Furthermore, the TnI bands were similar in sham animals in comparison with those of pigs subjected to reversible LAD ischemia. Thus, although TnI degradation can be demonstrated after irreversible injury in swine, it is not an essential feature of regionally stunned myocardium in vivo.

TnI Degradation in Isolated Langendorff Rat Hearts

We performed 6 additional experiments to confirm that the C5 monoclonal antibody detected TnI degradation as demonstrated using other TnI antibodies in the rat.[4,17] Figure 9 shows an immunoblot of LV protein from each of the hearts studied. Individual densitometric values and hemodynamics for each heart are provided in Table 6. No TnI degradation band was present in normal rat myocardium that was immediately excised and not subjected to buffer perfusion (n=2). After 20 minutes of global ischemia and 20 minutes of reperfusion at constant flow (n=2), LV end-diastolic pressure

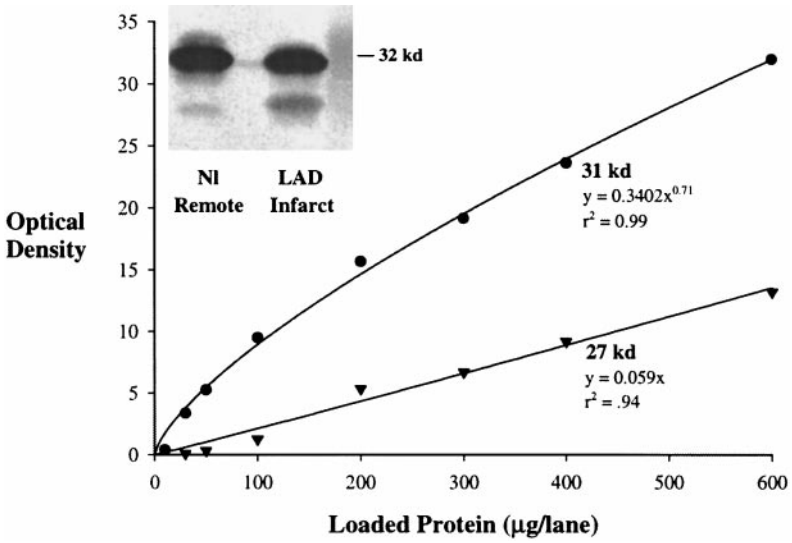

Figure 4. Positive in vivo control in a 24-hour-old porcine LAD myocardial infarct using the C5 monoclonal antibody. Inset (loaded at 200 μg/lane) shows that the low molecular mass TnI-immunoreactive band increased from 10% to 31% of the TnI band. The graph plots the optical density vs protein loading for both bands. The 27-kDa band was linearly related to protein concentration over a range from 0 to 600 μg/lane. In contrast, optical density of the 31-kDa TnI band was nonlinearly related to protein loading and underestimated TnI concentration above 50 μg/lane. Regression relations represent the best fit to the data. NI indicates normal.

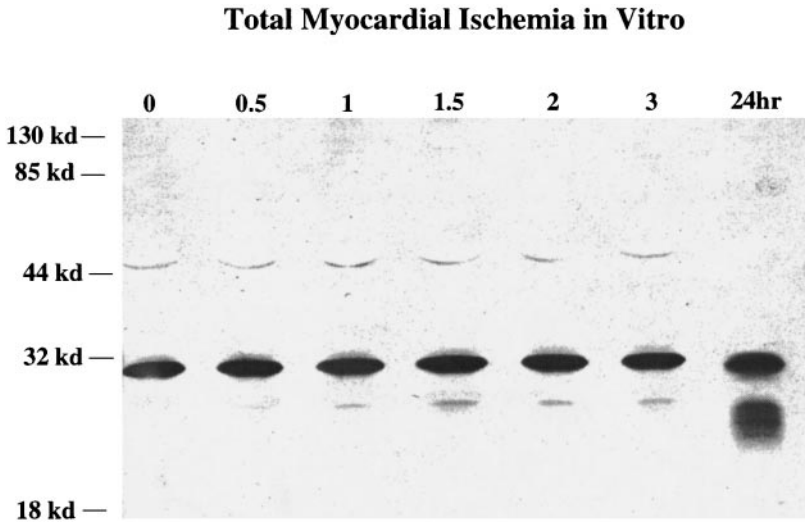

Figure 5. Western blot of TnI degradation after simulated total ischemia in isolated porcine myocardium in vitro. Fresh myocardial tissue was rapidly excised and incubated at 37°C. At selected times, samples were rapidly frozen for protein isolation and Western analysis, as described in Materials and Methods. Gels were loaded at 200 μg/lane, and immunoblotting was performed with the C5 monoclonal antibody. TnI degradation began at between 1 and 2 hours of global ischemia and was prominent at 24 hours, when multiple low molecular mass bands became apparent. The increase in the TnI degradation band commenced with the onset of irreversible injury (≈90 minutes) in this model.[15] Nonspecific binding to a higher molecular mass protein (≈45 kDa) could be demonstrated at the higher protein-loading conditions.

increased from 10 to 31 mm Hg, and systolic pressure fell from 89 to 66 mm Hg. As shown in Figure 9, a 27-kDa TnI degradation band appeared in reperfused hearts that averaged 12% of the TnI band by densitometry. Sham hearts perfused with buffer for 60 minutes maintained a constant level of LV systolic and end-diastolic pressure throughout the experiment, but this treatment had differential effects on TnI degradation. At normal filling pressures there was no TnI degradation band. However, when preload was elevated from 10 to 20 mm Hg to simulate levels encountered in ischemia/reperfusion, a degradation band appeared that was 17% of the TnI band despite the fact that LV systolic pressure was normal (112 mm Hg). These data indicate that the appearance of TnI degradation can be dissociated from ischemia and systolic dysfunction.

Discussion

Important new findings from our study include the following. First, dysfunctional myocardium after single episodes of reversible ischemia is not accompanied by alterations in mRNA levels for the SR Ca^{2+} ATPase or phospholamban, in contrast to increases in transcription of these proteins after repetitive episodes of ischemia in swine.[5,18] Second, unlike results in Langendorff buffer-perfused rat hearts, TnI is not degraded after reversible regional ischemia in swine in vivo. These findings fail to support the hypothesis that damage to SR uptake proteins or damage to TnI are essential features of stunned myocardium in swine.

Lack of TnI Degradation in Porcine Stunned Myocardium

Previous studies have raised the possibility that alterations in myofilament calcium sensitivity may be responsible for myocardial stunning.[19] In support of this, Gao et al[3] found evidence of selective TnI proteolysis in isolated, reperfused rat hearts subjected to 20 minutes of global ischemia. This was dependent on reperfusion and could be mimicked by in vitro incubation with the calcium-activated protease calpain I and blocked by inhibiting it with calpastatin. Although this supports the hypothesis that alterations in myofilament sen-

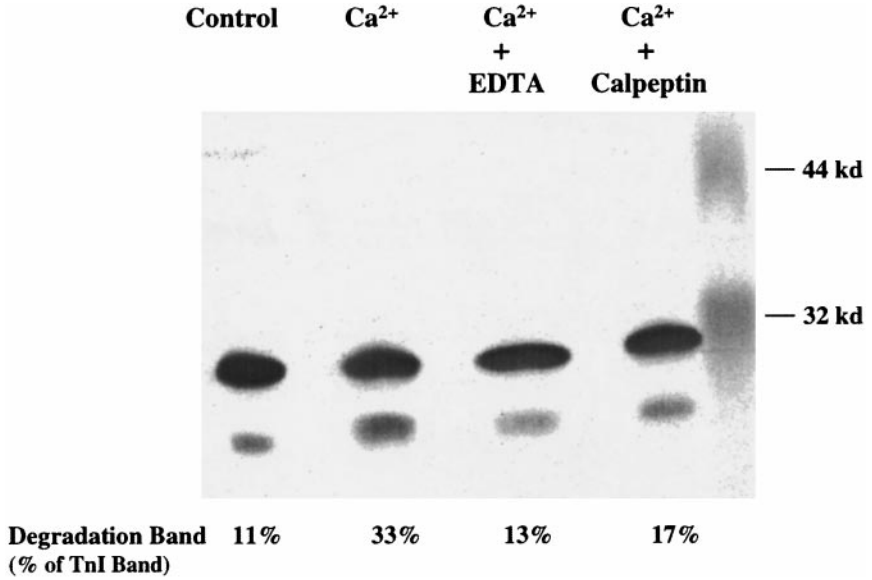

Figure 6. Western blot of TnI degradation after incubation of normal porcine myocardial protein with elevated calcium in vitro. Values below each gel are percentages of the 27-kDa band vs the 31-kDa TnI band. The left lane shows control myocardial protein incubated at 37°C for 60 minutes with a basal level of the degradation band. Myocardial protein in the other 3 lanes was incubated in 10 mmol/L calcium at 37°C. The degradation band increased from 11% to 33% in the presence of calcium. TnI degradation was blocked by incubation with EDTA or the calpain inhibitor calpeptin. These results indicate that TnI degradation from in vitro activation of a Ca^{2+}-dependent protease can be detected by Western analysis using the monoclonal antibody in porcine myocardium. Similar results were demonstrated at lower protein loading using the polyclonal TnI antibody (data not shown).

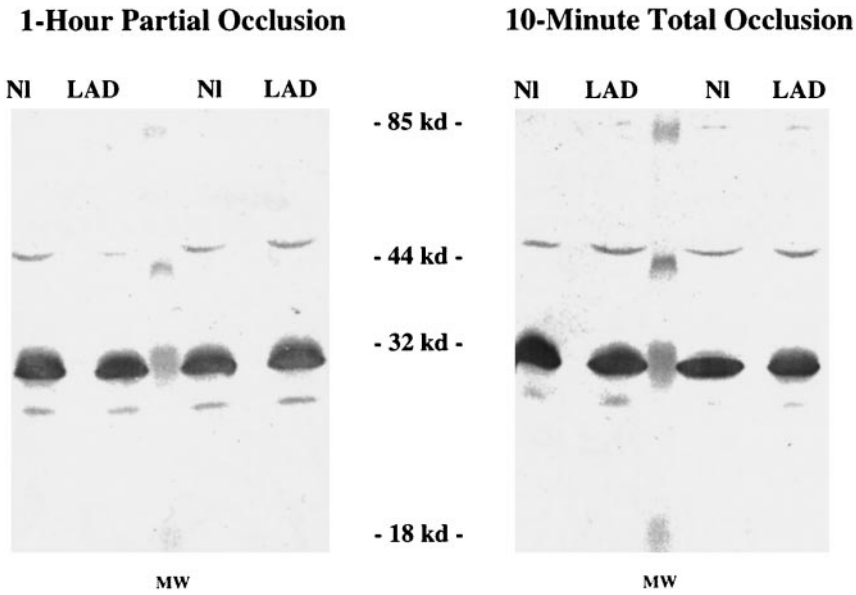

Figure 7. Western blot of TnI using the monoclonal antibody. Stunning after a 1-hour partial occlusion and 10-minute total occlusion did not produce any change in either the 31-kDa TnI band or the 27-kDa band in swine. Thus, TnI degradation was not present despite substantial regional dysfunction in the LAD region. NI indicates normal; MW, molecular weight markers.

sitivity may be due to alterations in TnI in rats, we found no evidence of TnI proteolysis after reversible regional ischemia in vivo in the pig with the use of either a polyclonal or monoclonal antibody for TnI. This was not related to the sensitivity of the antibodies to detect the degradation band in pigs, because activating endogenous proteases in vitro produced a marked increase in a 27-kDa TnI degradation band that could be blocked by EDTA or calpeptin. We also demonstrated TnI degradation in infarcted myocardium in vivo, as has previously been reported by others in dogs.[20] Finally, we failed to find an increase in the expression of TnI mRNA. Although this does not exclude the possibility that protein resynthesis could have occurred through other mechanisms, the fact that this occurs with a lack of a change in protein levels (either a reduction in the TnI band or increase in the TnI degradation band) makes it unlikely.

Ischemia-induced TnI degradation was first reported by Toyo-oka and Ross[20] in infarcted canine subendocardium. Westfall and Solaro[16] reported TnI loss and the appearance of a degradation band in rat myofibrillar protein preparations after simulated global ischemia for 1 hour in vitro. Although these authors argued that this time frame is associated with reversible injury in incubated canine tissue,[15] no independent assessment of viability was performed. Gao et al[3] found TnI proteolysis in buffer-perfused Langendorff rat hearts subjected to 20 minutes of global ischemia, but there was also no independent assessment of viability or enzyme release in the effluent, and the increase in the degradation band was not

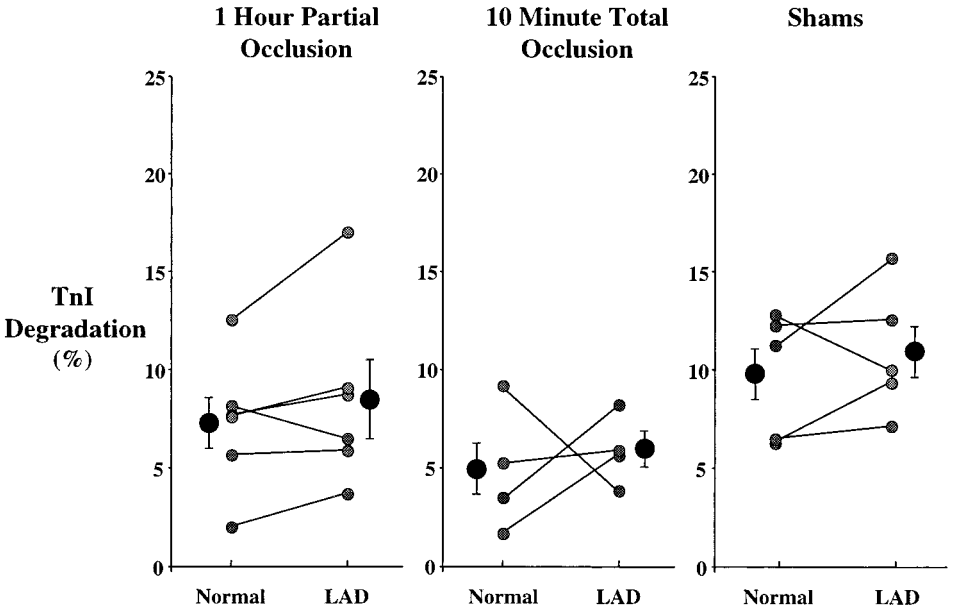

Figure 8. Paired densitometric analysis of TnI from all of the pigs studied. The 27-kDa band was present in normal myocardium and varied between 2% and 14% of the TnI band. Paired comparison demonstrated no differences between stunned (LAD) and normal myocardium, nor were there global changes as compared with sham controls. These data indicate that TnI loss and degradation are not findings associated with reversible ischemia in pigs. Gray circles depict individual measurements; black circles are averages±1 SE.

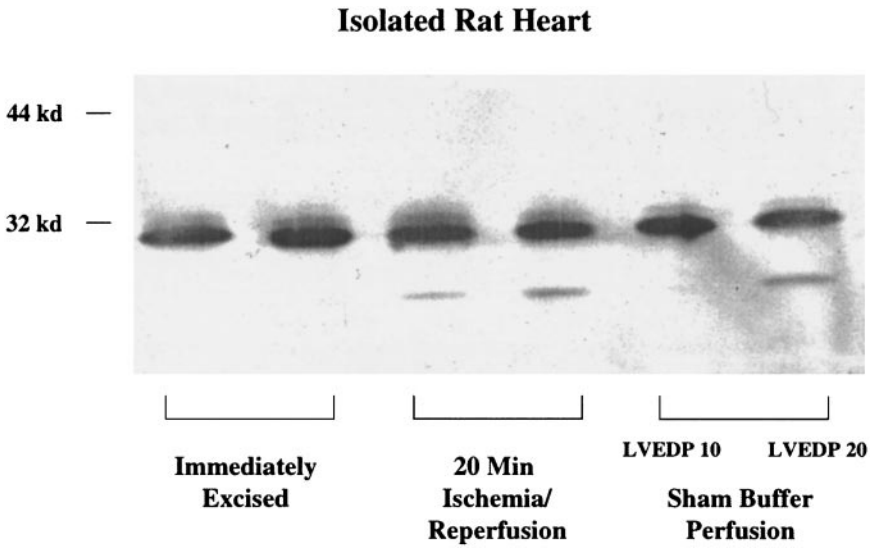

Figure 9. Western blot of TnI degradation in isolated Langendorff hearts perfused at constant flow. Each lane was loaded at a concentration of 200 μg of total protein and immunoblotting performed with the C5 monoclonal antibody. TnI degradation was not present in immediately excised rat heart tissue that was not subjected to buffer perfusion (lanes 1 and 2). In contrast, a degradation band appeared in isolated hearts subjected to 20 minutes of global ischemia and 20 minutes of reperfusion (lanes 3 and 4). Lanes 5 and 6 summarize TnI degradation in sham hearts subjected to 60 minutes of buffer perfusion. Although these hearts continued to develop pressure normally throughout the period of perfusion, a TnI degradation band could be produced by distending the left ventricle at an elevated (20 mm Hg) vs normal (10 mm Hg) preload.

quantified. Finally, Van Eyk et al[4] recently reported TnI proteolysis 45 minutes after ischemic durations of 15 and 60 minutes in the isolated, buffer-perfused rat heart subjected to reperfusion. In their study, TnI degradation was not quantified, but surprisingly, there was no reduction in TnI immunoreactivity, which, if anything, increased in stunned versus sham control hearts. Assessment of viability was qualitative using HPLC analysis of venous effluent. Protein was not detected in the effluent of shams, was least increased in the 15-minute occlusion group (1+), and was most elevated in the 60-minute group (3+).

Other studies have failed to identify TnI degradation after reversible ischemia in isolated hearts. Barbato et al[6] challenged the notion that the ischemia-induced bands were products of TnI. They failed to observe immunoreactivity of a 23-kDa band with 6 TnI antibodies and suggested that the 23-kDa band was actually αB-crystalline that had localized to the myofibrillar fraction in rats. Gorza et al[21] studied TnT and TnI immunoreactivity in the Langendorff guinea pig heart 30 minutes after it was subjected to 30 and 60 minutes of ischemia. They reported high molecular weight TnT and TnI complexes from calpain-mediated proteolysis on reperfusion but did not find a lower molecular weight degradation band for TnI. Matejovicova et al[22] found a lower molecular weight TnI-immunoreactive band that did not change after a 15-minute episode of global ischemia in isolated rabbit hearts. Finally, Lüss et al[23] and McDonald et al[24] recently failed to find TnI degradation after moderate ischemia in swine. None of these negative studies performed positive controls to confirm that the antibodies could detect TnI degradation in irreversibly damaged myocardium, which is a major limitation in interpreting the negative findings. Negative results may arise from species specificity of various TnI antibodies as well as variations in the epitope that may not be present in the TnI degradation band.[4,17]

Our positive controls are consistent with the appearance of TnI proteolysis in irreversibly injured myocardium in vivo as well as in vitro in swine. We also confirmed that both of the models of ischemia we used did not cause irreversible injury. None of the previous studies quantified the extent of necrosis, and it is possible that 15 to 20 minutes of global ischemia in buffer-perfused hearts subjected to constant flow may have led to irreversible injury coexisting with reversible stunning accounting for the appearance of TnI proteolysis. In support of this possibility are studies examining preconditioning in rat hearts which show that 57% of the area at risk is infarcted in

TABLE 6. Summary of Individual Measurements of TnI Degradation in Normal Rat Myocardium and in Isolated Buffer Perfused Rat Hearts

	Monoclonal TnI Antibody			LV Systolic Pressure, mm Hg		LV EDP, mm Hg	
	31 kDa, du	27 kDa, du	27 kDa/31 kDa, %	Initial	Final	Initial	Final
Rapidly excised	11.4	0	0	...	...	...	...
	10.6	0	0	...	...	...	...
20-minute ischemia/reperfusion	10.9	0.92	8.4	85	63	10	29
	10.4	1.57	14.9	92	68	10	32
Sham buffer perfusion							
Low EDP	12.9	0	0	97	92	10	10
High EDP	9.7	1.48	17.2	118	112	20	20

Densitometric measurements of TnI represent averages from duplicate gels (200 μg protein/lane) run under identical conditions. du indicates densitometric units; EDP, end-diastolic pressure.

control rats after a 20-minute LAD occlusion.[25] Data examining infarct size in buffer-perfused Langendorff rabbit hearts demonstrate that infarct size is 31% of the left ventricle after 20 minutes of ischemia.[26] These data support the possibility that necrosis rather than species variation may be a significant determinant of the difference in TnI degradation between swine and rats.

Data from our isolated rat heart experiments also raise another possibility to explain the differences in TnI degradation between global ischemia in vitro and regional ischemia in vivo. There was no low molecular mass band in normal rat hearts, but we were able to demonstrate TnI degradation after 20 minutes of ischemia/reperfusion, supporting the earlier findings of Gao et al[3] in rats with the use of an identical protocol. Nevertheless, the mechanism of TnI degradation may not be due to ischemia, because we were able to produce TnI degradation when LV end-diastolic pressure was elevated from 10 to 20 mm Hg in sham perfused hearts while systolic pressure remained normal. Because the Langendorff heart preparation is perfused at a constant LV volume, preload is markedly increased throughout reperfusion after ischemia. This may produce mechanical stretch that alters TnI and results in TnI proteolysis. Diastolic stretch has also been shown to induce myocyte apoptosis in isolated papillary muscles,[27] as well as to induce gene expression for proteins such as HSP-70 in Langendorff hearts.[28] Although further studies will be required to determine the relative role of irreversible injury and LV preload on findings in the rat, our results suggest limitations in extrapolating mechanisms from the in vitro buffer-perfused heart to the in vivo model of regional ischemia.

Although TnI proteolysis is not present in stunned myocardium in pigs, we cannot exclude the possibility that it may become an important determinant of contractile dysfunction in irreversible injury. In addition, the extent to which calpain activation (and presumably TnI proteolysis) can be blocked could affect the recovery of ventricular function by reducing irreversible injury and myocardial infarct size. Future studies will need to carefully evaluate the extent of irreversible injury to assess the role of myocyte salvage versus stunning on the recovery of contractile function, particularly when ischemic durations approaching the threshold for the onset of irreversible injury are used.

Myocardial Gene Expression After Single Episodes of Reversible Ischemia

An alternative hypothesis that has been set forth to explain contractile dysfunction after total coronary occlusions is altered SR calcium uptake and release. Although there is disparity in the effects of reversible ischemia on SR function in isolated vesicle preparations, with some showing it to be increased[29] whereas others show it to be reduced,[30] the expression of selected SR proteins is increased after repetitive coronary occlusions in pigs. The expression levels of the SR Ca^{2+} ATPase, phospholamban, and calsequestrin using Northern analysis and nuclear run-on assays were all variably increased after two 10-minute occlusions.[5,18] These changes were consonant with the notion that the dysfunction in stunned myocardium may be related to damage of selected SR proteins, with delayed recovery related to their resynthesis during reperfusion. Our findings after single episodes of ischemia argue against this interpretation. Although single episodes of reversible ischemia induced HSP-70 expression, partial occlusion and brief total occlusions were not accompanied by changes in the expression of any of the SR proteins examined.

Although the inability to assess temporal changes in protein and mRNA is a potential limitation of our experiments, it is an unlikely explanation for the absence of protein or mRNA changes after 60 minutes of reperfusion for several reasons. First, Lüss et al[23] recently reported no alteration in mRNA or protein for TnI in pigs subjected to 90-minute ischemia, where samples were analyzed at an earlier time point (30 minutes) after reperfusion. Second, whereas we, like Gao et al,[3] found TnI degradation as early as 20 minutes after reperfusion in the isolated, buffer-perfused heart, Van Eyk et al[4] and McDonough et al[17] showed that TnI degradation in rats persisted at 45 minutes of reperfusion. Finally, the TnI degradation band in swine persisted in vivo for a considerable duration of time as evidenced by the prominent band we found in our infarcted control hearts studied 24 hours after myocardial ischemia (Figure 4).

In summary, the results of our study demonstrate that single episodes of reversible regional ischemia in swine are sufficient to cause pronounced stunning and induce mRNA levels of HSP-70. Nevertheless, neither brief total occlusions nor prolonged partial occlusions produce TnI proteolysis or induce SR gene expression. Whereas TnI proteolysis occurs in irreversibly injured myocardium in the pig and can be simulated by activating Ca^{2+}-activated proteases, it is not a feature of reversibly injured myocardium in vivo. To determine whether resynthesis of other reversibly damaged components of the contractile apparatus is responsible for the delayed recovery of function in stunned myocardium in pigs will require further study.

Acknowledgments

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Absence of Troponin I Degradation or Altered Sarcoplasmic Reticulum Uptake Protein Expression After Reversible Ischemia in Swine

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