

Preload Induces Troponin I Degradation Independently of Myocardial Ischemia

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Background—Although global ischemia induces troponin I (TnI) degradation, regional ischemia does not. We hypothesized that this disparity is related to preload-induced proteolysis, which varies as a function of the amount of myocardium at risk of ischemia.

Methods and Results—Isolated rat hearts were buffer-perfused at controlled levels of preload. Increasing preload to 25 mm Hg in the absence of ischemia produced pronounced TnI degradation (27 kDa versus 31 kDa bands: $16.4 \pm 3.6\%$ versus $4.7 \pm 1.9\%$ in immediately excised controls, $P < 0.05$). TnI degradation could be blocked by preventing the activation of endogenous calpains with 25 $\mu\text{mol/L}$ calpeptin ($4.3 \pm 0.6\%$). This improved function, with left ventricular systolic pressure increasing from 103 ± 4 mm Hg to 137 ± 7 mm Hg ($P < 0.05$). Eliminating elevations in preload after global ischemia-induced stunning also prevented TnI degradation.

Conclusions—Calpain-mediated TnI proteolysis can be dissociated from stunning and arises from elevations in preload rather than ischemia. This raises the possibility that ongoing preload-induced TnI degradation could impair myocardial function long-term. (*Circulation*. 2001;103:2035-2037.)

Key Words: troponin I ■ calpain ■ myocardial stunning ■ ischemia

The role of troponin I (TnI) proteolysis as a mechanism of myocardial stunning is controversial. TnI degradation occurs after reversible global ischemia in the Langendorff rat heart,¹⁻³ and overexpressing degraded TnI in an amount similar to that observed after ischemia results in global contractile dysfunction in mice.⁴ Nevertheless, TnI degradation is absent in regionally stunned myocardium in swine³ and dogs.⁵ Antibody immunoreactivity is not an explanation, because marked TnI degradation can be demonstrated after irreversible injury.^{3,6,7}

An alternative mechanism that would reconcile these discordant findings is that marked elevations in left ventricular (LV) end-diastolic pressure (EDP) after global ischemia in the Langendorff heart (>30 mm Hg) causes TnI degradation. We hypothesized that preload may produce mechanical strains that lead to myocyte calcium entry⁸ and subsequent activation of μ -calpains to produce TnI proteolysis^{1,6} independently of ischemia. To test this, we subjected hearts to elevated preload in the absence of ischemia and we evaluated whether ischemia-induced TnI degradation could be blocked by lowering preload after global ischemia. The results demonstrate that preload induces calpain-mediated TnI degradation independently of ischemia.

Methods

Protocols were approved by the Institutional Animal Care and Use Committee. Hearts excised from male Sprague-Dawley rats (200 to

250 g) anesthetized with ether and anticoagulated with heparin (2000 U/kg IV) were retrogradely perfused at 65 mm Hg with Krebs-Henseleit buffer equilibrated with 95% O₂ and 5% CO₂. Buffer included (in mmol/L): NaCl 118, NaHCO₃ 25, KHPO₄ 1.2, KCl 4.7, MgSO₄ 1.2, CaCl₂ 1.5, and glucose 11.0. Temperature was maintained at 37°C. Hearts were paced at 280 bpm except during ischemia, in which pressure development ceased and pacing was stopped. Coronary flow was measured by timed effluent collection. Isovolumetric measurement of function was made using a balloon inserted into the left ventricle. Protocols began after a 20-minute equilibration period during which LVEDP was set to 10 mm Hg.

Experimental Protocols

First, we determined whether preload induces TnI degradation independently of ischemia. In one group ($n=10$), the balloon was inflated to increase LVEDP from 10 to 25 mm Hg for 40 minutes. In a second group ($n=8$), the calpain inhibitor calpeptin (25 $\mu\text{mol/L}$) was added to the buffer 5 minutes before elevating LVEDP to 25 mm Hg for 40 minutes. Rapidly excised hearts were used as controls ($n=9$).

Next, we determined whether ischemia produced TnI degradation independently of elevated preload. The control group was buffer-perfused at an LVEDP of 10 mm Hg for 60 minutes ($n=10$). In the experimental group ($n=10$), preload was set to 10 mm Hg during the initial 20 minutes of perfusion. We then occluded inflow for 20 minutes and deflated the LV balloon to keep preload <10 mm Hg for the remainder of the experiment. After 20 minutes of reperfusion, LVEDP was briefly returned to 10 mm Hg to document that LV systolic pressure (LVSP) was depressed in a manner consistent with stunning.

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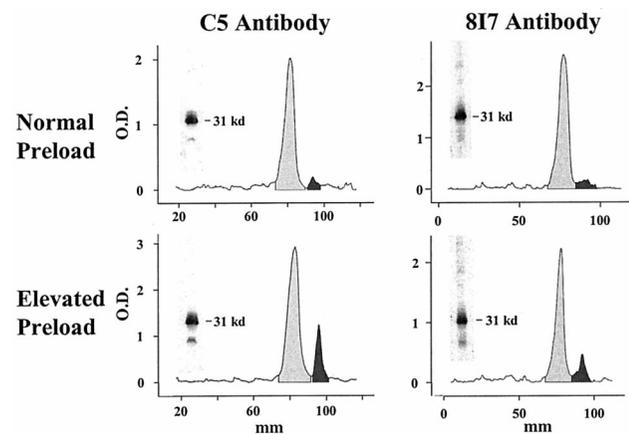


Figure 1. Examples of buffer-perfused hearts at normal (10 mm Hg) and elevated (25 mm Hg) preload. Elevating preload produced TnI degradation in the absence of ischemia. Profile analysis was performed along the length of each gel, and the background was subtracted from the area under each peak. The area of the low-molecular-weight bands (dark shading) were compared with the major TnI band (light shading) and expressed as a percent. High-molecular-weight TnI cross-linking products were not produced in any of the protocols.

Western Analysis

Protein isolation and immunoblotting were performed on flash-frozen samples as previously described.³ To insure accurate quantitation and linearity of the TnI degradation band at low concentrations, 12% SDS-polyacrylamide gels were loaded with 200 μ g of protein per lane.³ Gels were transferred to Immobilon-P membranes and soaked in 3% nonfat dry milk in PBS for 1 hour. After rinsing, gels were incubated for 1 hour with a bovine TnI monoclonal antibody (clone C5, Biogenesis; 1:2000 dilution). They were then rinsed and incubated for 1 hour with horseradish peroxidase protein G (1:10 000 dilution). TnI immunoreactivity was visualized with 3,3',5,5'-tetramethylbenzidine membrane peroxidase substrate. Samples were also assayed using a human cardiac TnI monoclonal antibody (8I7, Spectral Diagnostics; 1:5000 dilution). Immunoreactivity was assessed with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10 000 dilution). Both TnI antibodies were previously shown to cross-react in rats.^{2,3}

Data are presented as mean \pm SEM. Hemodynamics were analyzed by ANOVA followed by the Student-Neumann-Keuls test. Group *t* tests were used to compare TnI degradation among experimental groups; $P < 0.05$ was considered significant.

Results

Figure 1 shows representative immunoblots for TnI using both antibodies. Elevating preload produced TnI degradation. A weak, lower weight immunoreactive band was seen at 27 kDa with each antibody in nearly all of the hearts, including those that were immediately excised. Summary results were based on background-subtracted integrated density of the respective bands (shaded areas) along the width of each lane. The 27 kDa band was normalized to the 31 kDa TnI band and expressed as a percent.

Effects of Increased LVEDP in the Absence of Ischemia

Elevating preload from 10 to 25 mm Hg increased LVSP from 81 ± 3 to 103 ± 4 mm Hg ($P < 0.05$), with no change in LV dP/dt (Table 1). As shown in Figure 2, this increased TnI degradation from $4.7 \pm 1.9\%$ in immediately excised myocardium to $16.4 \pm 3.6\%$ ($P < 0.05$). TnI degradation was completely blocked when preload was elevated in the presence of calpeptin

TABLE 1. Hemodynamics in Animals Subjected to Increased Preload in the Absence of Ischemia

	Elevated Preload		Calpeptin	
	Initial	Final	Initial	Final
LVEDP, mm Hg	10	25	10	25
LVSP, mm Hg	81 ± 3	$103 \pm 4^*$	87 ± 4	$131 \pm 7^*$
dP/dt max, mm Hg/s	2009 ± 73	1862 ± 65	2108 ± 69	$3277 \pm 245^*$
-dP/dt max, mm Hg/s	1636 ± 115	1608 ± 105	1867 ± 152	2106 ± 213
Coronary flow, mL/min	12 ± 0.7	11.5 ± 0.6	12.5 ± 0.5	14.0 ± 0.8

Values are mean \pm SEM.

* $P < 0.05$ vs initial.

($4.3 \pm 0.6\%$, $P = NS$ versus excised hearts). Blockade of preload-induced TnI degradation with calpeptin improved LV function (LVSP, 137 ± 7 mm Hg versus 103 ± 4 mm Hg, $P < 0.05$; LV dP/dt, 3277 ± 245 mm Hg/s versus 1862 ± 65 mm Hg/s, $P < 0.05$). Results with the 8I7 antibody were similar to those with the C5 antibody (Figure 2).

Effects of Global Ischemia With Normal Preload

As summarized in Table 2, hearts subjected to 20 minutes of ischemia with the left ventricle vented demonstrated stunning after 20 minutes of reperfusion (LVSP, 61 ± 4 mm Hg versus 82 ± 3 mm Hg, $P < 0.05$). In buffer-perfused controls, TnI degradation averaged $8.5 \pm 2.5\%$ (Figure 2). Despite myocardial stunning, there was no change in TnI degradation after

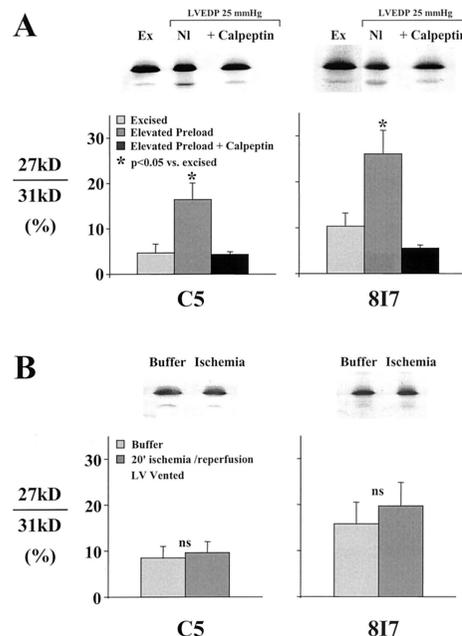


Figure 2. Summary of TnI degradation in the 2 experimental groups. Densitometric measurements of the TnI degradation band are summarized for the C5 antibody on the left and for the 8I7 antibody on the right. Elevating preload in the absence of ischemia (A) increased TnI degradation compared with excised controls. Elevation could be completely blocked with the μ -calpain inhibitor calpeptin. When elevations in preload were prevented after global ischemia (B), stunning developed, but there was no increase in TnI degradation compared with buffer-perfused control hearts. Thus, TnI degradation was secondary to elevations in preload and was not associated with myocardial stunning.

TABLE 2. Hemodynamics in Animals Subjected to 20 Minutes of Ischemia Versus Buffer-Perfused Controls

	Buffer		20 min of IS/RP	
	Initial	Final	Initial	Final
LVEDP, mm Hg	10	10	10	10
LVSP, mm Hg	83±3	80±3	82±3	61±4*
dP/dt max, mm Hg/s	1928±115	2014±134	1873±87	1382±92*
-dP/dt max, mm Hg/s	1684±124	1472±146	1726±93	1102±60*
Coronary flow, mL/min	12.5±0.5	11.5±0.8	12.0±0.6	11.5±0.5

Values are mean±SEM. IS/RP indicates ischemia/reperfusion.

* $P < 0.05$ vs. initial.

ischemia ($9.6 \pm 2.4\%$, $P = \text{NS}$). Results with the 8I7 antibody were similar to those with the C5 antibody.

Discussion

There are several important new findings in our study. First, increasing preload to a level that is frequently encountered in pathophysiological states such as heart failure produces TnI degradation. This is functionally significant and seems to be mediated by the activation of endogenous proteases that can be blocked by the μ -calpain inhibitor calpeptin. Second, TnI degradation after global ischemia can be eliminated by preventing excessive elevations in preload during reperfusion. Thus, proteolysis of TnI is the result of increased diastolic pressure and is dissociated from myocardial stunning.

Although TnI proteolysis occurs after irreversible injury,^{3,6,7,9} Gao and colleagues¹ demonstrated that it also occurs after reversible global ischemia in the isolated heart. Others^{2,3} have reproduced this finding in Langendorff hearts using a variety of TnI antibodies. Unlike in vivo models of regional ischemia, the globally stunned, isovolumic heart is subjected to marked increases in LVEDP during reperfusion (>30 mm Hg). Our results show that increased diastolic pressure is sufficient to cause TnI degradation independently of ischemia. Furthermore, function at an elevated preload improved after preventing TnI degradation with calpeptin. Systolic LV pressure increased by 27% and LV dP/dt increased by 47% compared with buffer perfusion alone, indicating an improvement in LV performance that was related to the preservation of intact TnI. Although we cannot exclude the possibility that this peptide inhibitor alters LV function through unidentified nonspecific mechanisms, the findings raise the possibility that long-term alterations in LVEDP characteristic of advanced or decompensated congestive heart failure can produce global TnI degradation. Although speculative, this may contribute to chronic contractile dysfunction in a fashion similar to transgenic mice overexpressing degraded TnI.⁴

The failure of global ischemia to induce TnI degradation when preload elevation is prevented is consonant with the absence of increased TnI degradation after regional ischemia in swine and dogs.^{3,5} The apparent role of TnI degradation as a mechanism of stunning is not related to species differences but to important physiological differences arising from the experimental preparation. Thus, although preload-induced TnI degradation and postischemic

stunning occur in the globally ischemic rat heart, they are distinct phenomenon that are not causally related.

Methodological Limitations

Although we did not assess transmural flow, the existence of subendocardial ischemia when preload was increased is unlikely because coronary outflow did not decrease and LV pressure increased rather than decreased. Furthermore, our previous study failed to produce TnI degradation when regional subendocardial flow was reduced to 32% of baseline values for 1 hour.³

Clinical Implications

Interestingly, myocardial TnI degradation has frequently been demonstrated in the absence of ischemia in humans,¹⁰ and serum TnI elevations that are independent of ischemia have been reported in fluid overload states such as renal failure¹¹ and advanced heart failure.¹² Serum TnI elevation also occurs frequently in humans with acute coronary syndromes in the absence of other biochemical markers of injury. Although speculative, these may reflect preload-induced myocyte calcium entry and TnI proteolysis, which may be preludes to stretch-induced myocyte apoptosis.^{9,13} Further studies will be required to determine whether preventing TnI degradation with calpain inhibitors can prevent the progression of left ventricular dysfunction.

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