Transgenic Mouse Model of Stunned Myocardium

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Stunned myocardium is a syndrome of reversible contractile failure that frequently complicates coronary artery disease. Cardiac excitation is uncoupled from contraction at the level of the myofilaments. Selective proteolysis of the thin filament protein troponin I has been correlated with stunned myocardium. Here, transgenic mice expressing the major degradation product of troponin I (TnI_{1-193}) in the heart were found to develop ventricular dilatation, diminished contractility, and reduced myofilament calcium responsiveness, recapitulating the phenotype of stunned myocardium. Proteolysis of troponin I also occurs in ischemic human cardiac muscle. Thus, troponin I proteolysis underlies the pathogenesis of a common acquired form of heart failure.

Coronary artery disease remains the leading cause of death in Western society, despite major strides in the treatment of acute coronary occlusion and myocardial infarction. The goal of therapy in these syndromes is prompt reestablishment of coronary artery patency, and thus, myocardial perfusion. Although the extent of irreversible cardiac damage can be effectively decreased, patients often suffer cardiac dysfunction lasting for one to several days. This common clinical scenario of reversible postischemic contractile dysfunction is referred to as myocardial stunning (*1*). The pathogenesis of stunning remains unclear. Analysis of experimental models has localized the lesion to the contractile machinery, with the key phenotypic features being a diminution of myofilament calcium responsiveness and a decrease in maximal activation (*2*). Such dysfunction is correlated with partial proteolysis of the thin filament regulatory protein troponin I (TnI) (*3*).

To determine if TnI modification causes stunned myocardium, we attempted to recapitulate the disorder in transgenic mice. The major proteolytic product of TnI in stunned myocardium (TnI_{1-193}) is missing the 17 COOH-terminal residues (*4*). We created three independent lines of transgenic mice expressing TnI_{1-193} in the heart driven by the murine α-myosin heavy chain (α-MHC) promoter (Fig. 1A) (*5*). Southern blotting indicated that the founders had 9 to 13 copies of the transgene. Western analysis of heart myofibril preparations (Fig. 1B) revealed expression of TnI_{1-193} at 9 to17% of the level of endogenous TnI (*6*). All three lines were able to reproduce, and unstressed mice were viable until at least 12 months of age without evidence of overt heart failure (*7*). Transgenic mice exhibited cardiac enlargement (Fig. 1C) and echocardiography (*8*) established that this was due to ventricular chamber dilatation (Fig. 1D). The heart walls were not hypertrophied (Fig. 1D), despite a modest (4.2 ± 0.3 versus 3.7 ± 0.3 mg/g, $P = 0.04$) increase in heart-to-body-weight ratio in age-matched transgenic versus nontransgenic mice. TnI_{1-193} mice did not have increased mRNA content of myocardial hypertrophy markers β-MHC, α-skeletal actin, and atrial natriuretic factor, nor was the expression of phospholamban or sarcoplasmic reticulum adenosine triphosphatase (ATPase) significantly decreased (*9*). Histologic analysis (*10*) revealed normal myocellular architecture (Fig. 1E), consistent with the phenotype of stunned myocardium (*1*), but in sharp contrast to the myocyte disarray and fibrosis in other mouse models of cardiomyopathy (*11*). James *et al.* have recently characterized transgenic lines that express a cardiac TnI mutant, R145G ($Arg^{145} \rightarrow Gly^{145}$), found in human hypertrophic cardiomyopathy (HCM) (*12*). The lines expressing the highest amounts of R145G had early lethality with severe myocardial fibrosis and disarray, whereas lines expressing lower amounts demonstrated hy-

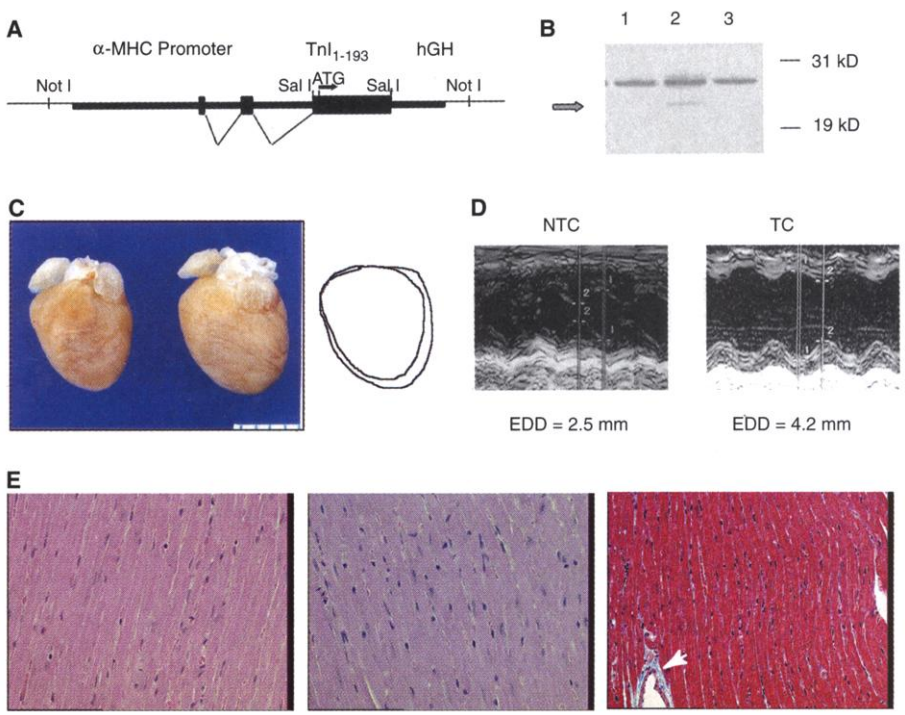

Fig. 1. Transgenic model with cardiac expression of truncated troponin I. (**A**) The construct used to generate the transgenic mice is shown. Details are described in (*5*). (**B**) Immunoblot of modified myofibril preparations of cardiac tissue from a transgenic mouse (lane 2) and two nontransgenic siblings (lanes 1 and 3). Molecular weight markers are indicated at right and the arrow indicates the truncated troponin I in the transgenic mouse heart. (**C**) Gross photograph of hearts from a nontransgenic (left) and transgenic littermate (right). The transgenic heart is enlarged and has a globular shape compared to the nontransgenic heart, as illustrated by the overlapped outlines of the ventricles. (**D**) m-mode echocardiogram of a nontransgenic and transgenic heart illustrating the left ventricular chamber dilatation of the transgenic heart. EDD is the end diastolic dimension of the left ventricular chamber. (**E**) Representative hematoxylin and eosin stains of myocardium from nontransgenic (left) and transgenic (middle) mice. The right panel is a Masson's trichrome stain of the transgenic heart and illustrates the normal blue staining of perivascular connective tissue (arrow) with the remainder of tissue free of fibrosis.

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percontractility and abnormal diastolic relaxation as well as fibrosis. Thus, the TnI_{1-193} mice have a distinct phenotype from that produced by a TnI HCM mutant. However, it should be recognized that allelic variants of HCM may have widely variable phenotypes; thus, our model may have some relevance to inherited disorders of the myofilaments.

Ventricular function was assessed in vivo with simultaneous measurement of left ventricular chamber pressure-volume relationships in order to derive indices of ventricular function independent of the preload imposed by ventricular filling or afterload imposed by the resistance of the systemic arterial system (*13, 14*). The TnI_{1-193} mice exhibited dilated left ventricles as illustrated by in vivo measurements of ventricular volume (Table 1 and Fig. 2A). Systolic function was markedly depressed in the TnI_{1-193} mice as reflected by the decrease in the rate of pressure development (dP/dt_{max}) and by reductions in the load-independent indices of preload-recruitable stroke work (PRSW) and end-systolic elastance (Ees) (Fig. 2C). Diastolic relaxation was also slowed (Table 1). Heart rate was slightly slower in the transgenic mice, but this change was too small to explain the marked abnormalities in contractile function. All these features recapitulate genuine myocardial stunning, in which there is both systolic and diastolic dysfunction (*1*).

Stunned myocardium remains responsive to the augmentation of contractility by β-adrenergic agonists (*15*). Isoproterenol (administered at 0.5 to 4.5 μg/kg body weight/min) increased dP/dt_{max} to a mean of 156 ± 28% of the base line in four transgenic mice. The adrenergic effect on dP/dt_{min} was not augmented in these hearts (mean 102 ± 8% of the base line), a pattern similar to the response in mice in which cardiac TnI is replaced by its slow twitch isoform that lacks sites for protein kinase A (PKA) (*16*). Although the PKA sites reside in the NH_2-terminal portion of TnI, interactions between the COOH- and NH_2-termini and/or altered basal phosphorylation could explain the blunted effect of adrenergic stimulation on ventricular relaxation in TnI_{1-193} mice.

We investigated calcium cycling and contractile activation in ventricular trabeculae from transgenic mice and nontransgenic siblings (*17*). Calcium transients were not reduced in muscles from TnI_{1-193} mice (Fig. 3A), despite the reduction in contractile force. Steady-state analysis identified a decrease of maximal calcium-activated force and a rightward shift in the force-calcium relationship as the bases of the diminished contractility (Fig. 3B). Such changes, which indicate a lesion of myofilament calcium activation, verify that the truncation of TnI is sufficient to produce the cellular pathophysiology of stunned myocardium.

Multiple studies of excitation-contraction coupling in animal models of stunning have revealed that calcium cycling is unimpaired (*2*). However, stunning diminishes in vitro cardiac muscle tension or in vivo myocardial wall thickening in rat, ferret, dog, baboon, and porcine models of stunned myocardium (*2, 18*). Recently, there has been controversy

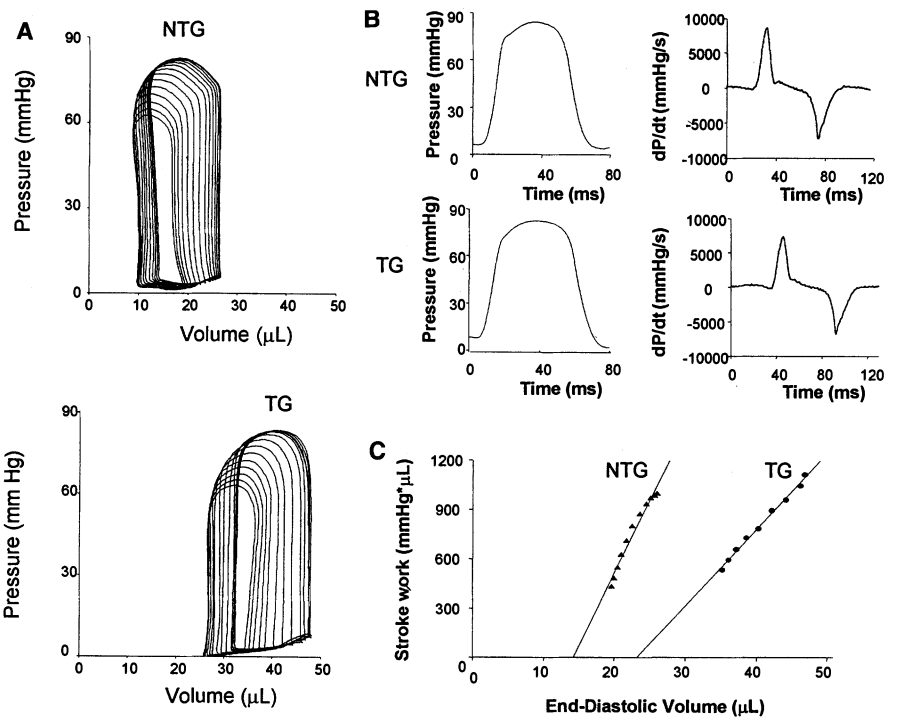

Fig. 2. Left ventricular mechanics measured in vivo. (**A**) Pressure-volume loops from representative nontransgenic (NTG, top) and transgenic (TG, bottom) mice measured during a transient reduction in cardiac preload. The TG mouse has an increased ventricular volume and a decrease in end-systolic elastance (slope of line intersecting left upper aspect of loop), indicating decreased systolic function. (**B**) Left ventricular pressure tracing (left panels) from NTG and TG mice. The right panels illustrate the first derivative of ventricular pressure (dP/dt) in NTG and TG mice demonstrating a decreased dP/dt_{max} (positive deflection) and dP/dt_{min} (negative deflection) in the TG. (**C**) Relationship of stroke work versus end-diastolic volume in representative NTG and TG mice. The slope of this relationship is the preload-recruitable stroke work (PRSW), a load-independent measure of systolic function.

Table 1. Characterization of left ventricular mechanics with impedance micromanometer catheter. Means are ± SD with *P* values from *t* -test. *P* values >0.05 are considered nonsignificant (NS). ESP and EDP are end-systolic and end-diastolic pressures. ESV and EDV are end-systolic and end-diastolic volume. CO and CI are cardiac output and index. The dP/dt_{max} is the maximal rate of pressure development and dP/dt_{min} is the maximal rate of decay of pressure. Ees is end-systolic elastance. PRSW is preload recruitable stroke work, the ratio of stroke work to end diastolic volume. Tau is the mono-exponential time constant of relaxation.

Parameter	Nontransgenic (n = 5)	Transgenic TnI_{1-193} (n = 7)	*P* value
	Hemodynamics		
Heart rate (min^{-1})	602 ± 35	517 ± 52	0.01
ESP (mmHg)	96.4 ± 19.2	79.9 ± 8.3	NS
EDP (mmHg)	5.0 ± 1.5	6.6 ± 1.3	NS
ESV (μl)	14.4 ± 4.4	28.0 ± 9.0	0.01
EDV (μl)	31.3 ± 9.1	50.5 ± 13.4	0.02
CO (ml/min)	10.0 ± 3.5	10.8 ± 3.7	NS
CI (ml/min)	0.30 ± 0.10	0.37 ± 0.13	NS
	Systolic function		
dP/dt_{max} (mmHg/s)	11,112 ± 2,022	7,409 ± 1,948	0.01
Ees (mmHg/μl)	16.0 ± 7.6	4.5 ± 1.0	0.005
PRSW (mmHg)	90.2 ± 6.4	64.4 ± 16.3	0.008
	Diastolic function		
dP/dt_{min} (mmHg/s)	10,844 ± 2,885	7,254 ± 1,080	0.01
Tau (ms)	4.7 ± 0.8	6.1 ± 0.6	0.007

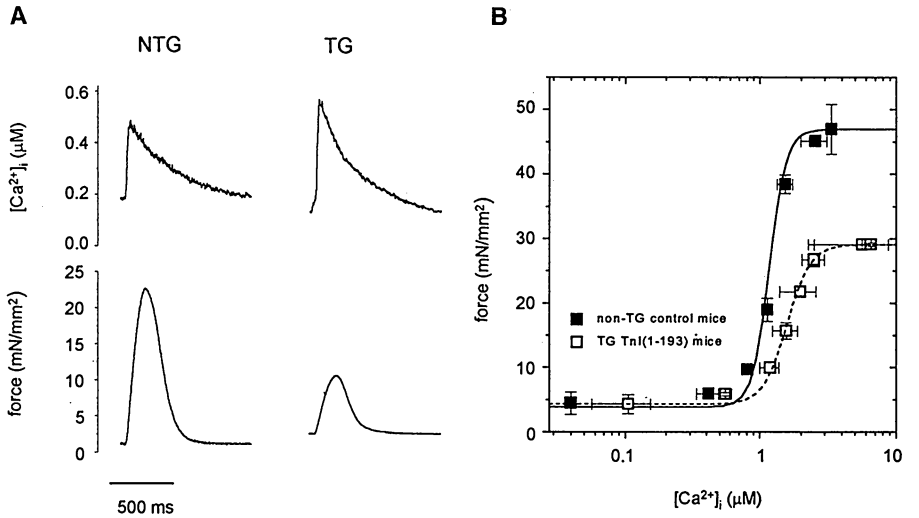

Fig. 3. Measurements of calcium and force dynamics in intact trabecular muscle. (**A**) Representative calcium transients from NTG and TG mice. The twitches were produced by field stimulation with external calcium concentration $[Ca^{2+}]_o$ of 2 mM at 22°C. Data indicated no significant difference in the mean calcium transient amplitudes between TG and NTG mice (0.62 ± 0.31 μM versus 0.41 ± 0.13 μM). The lower panel illustrates the simultaneous twitch tension correlating with the calcium transients in the upper panel. Pooled data indicated a tendency for the twitch tension to be lower in the TG mice, although this difference was not statistically significant (NTG 15.3 ± 4.56 mN/mm² versus TG 10.33 ± 6.05 mN/mm²). (**B**) Steady-state force-calcium relationship. The maximal calcium-activated force is significantly greater in the NTG mice, (46.95 ± 7.77 mN/mm² in four NTG versus 29.10 ± 0.44 mN/mm² in three TG mice, $P < 0.02$). There is a rightward shift in the half-maximal effective concentration (EC_{50}) from 1.17 ± 0.41 μM in NTG mice to 1.58 ± 0.63 μM in TG mice. The Hill coefficient was also decreased from 7.01 ± 2.11 to 5.0 ± 2.72 in the TG mice consistent with a decrease in cooperativity.

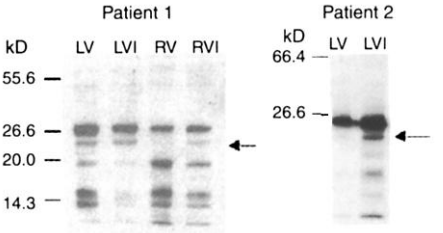

Fig. 4. Immunoblots of myocardial samples from right ventricle (RV) and left ventricle (LV) of two patients undergoing coronary artery bypass surgery for ischemic disease. Samples were obtained before (LV, RV) and after (LVI, RVI) total global ischemia induced by aortic cross-clamping and cardioplegic cardiac arrest. TnI and proteolyzed fragments were identified using epitope-specific antibodies, and analyzed by electrophoretic mobility. The TnI fragment indicated by the arrows was also demonstrated to have lost reactivity to COOH-terminal–specific TnI antibody (*28*) and is comparable in size to the rat cardiac TnI_{1-193} product. Additional degradation products are discussed in the text. Samples from patient 1 were also probed with antibodies against α-actin and myosin light chain 1, and no proteolysis of these proteins was detected (*28*).

as to whether the modification of TnI is consistently detected in large-animal models of stunning (*19*), bringing into question whether experimental models are pertinent to human postischemic cardiac dysfunction. To address this issue, we examined human myocardial samples (Fig. 4), obtained from patients undergoing coronary bypass surgery for ischemic disease (*20*). These blots reveal TnI proteolysis similar in spectrum to a rat model of ischemic injury (*3, 4*). In the rat, brief periods of ischemia followed by reperfusion result in partial proteolysis of TnI to TnI_{1-193} without evidence of cellular necrosis or proteolysis of other sarcomeric proteins (*3*). However, with increasing length of ischemia, there is further proteolysis of TnI with additional smaller TnI fragments (*3, 4*). Prolonged ischemia may also result in cellular necrosis and ultimately release of proteolyzed TnI and other sarcomeric proteins into serum. The human samples (Fig. 4) were obtained from patients who required surgery for ischemic disease, and thus it is not surprising that in some cases there is significant proteolysis of TnI even before the global ischemia induced by aortic cross-clamping. However, the product equivalent to rat TnI_{1-193} is prominent in the postischemic myocardium, and the lower molecular weight TnI products may decrease, perhaps due to necrosis of cells with severe ischemic damage. Importantly, TnI proteolysis may be observed before aortic cross-clamping even in the absence of serum markers of myocardial necrosis (*21*).

The reperfusion that follows brief episodes of ischemia is accompanied by transient elevation of intracellular calcium, and it has been proposed that this activates the calcium-dependent protease calpain I, leading to the truncation of TnI (*1*). Here, we show that the presence of truncated TnI, independent of ischemia and reperfusion, fully recapitulates the stunned phenotype. TnI acts as the molecular switch for contraction and relaxation of muscle. During diastole, when $[Ca^{2+}]_i$ (intracellular calcium concentration) is low, the inhibitory region of cardiac TnI (residues 137 to 148) interacts strongly with actin-tropomyosin (actin-Tm) and maintains the thin filament in a "closed" state in which the cross-bridges are weakly bound but nonforce generating, or in a "blocked" state in which cross-bridge formation is sterically hindered (*22*). With increased $[Ca^{2+}]_i$ during systole, interactions between TnI and troponin C (TnC) increase, and this results in the rearrangement of actin-Tm to an "open" state, promoting cross-bridge formation and contraction. Additional regions of TnI modulate the interaction with both actin-Tm and TnC and result in long-range structural changes within troponin itself as well as along the thin filament. Human TnI mutants associated with HCM have been noted both in the inhibitory region and in the COOH-terminal region of TnI (*23*). One of two known TnI HCM mutations in the extreme COOH-terminus is associated with predominantly apical hypertrophy (*23*). In vitro studies suggest that the COOH-terminus of cardiac and skeletal TnI contains additional binding sites for actin-tropomyosin and/or troponin T (TnT), whereas residues at or just upstream of the cleavage site form a region that interacts with the NH_2-terminus of troponin C (*24, 25*). However, the mechanism by which TnI truncation decreases maximal force is unknown (*26*).

Human genetic studies have established that mutations and deletions in contractile protein genes can produce dilated or hypertrophic cardiomyopathies (*27*). In contrast to these inherited cardiomyopathies that are relatively uncommon, myocardial stunning is a common form of contractile dysfunction. Our findings highlight the concept that posttranslational modifications of proteins involved in excitation-contraction coupling may play a key role in cardiac conditions such as ischemic injury or heart failure. The finding of TnI proteolysis in human ischemic myocardium suggests that therapy for stunned myocardium should be directed toward preventing or compensating for the myofilament defect evident in the TnI_{1-193} mice.

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5. The murine α-MHC construct [A. Subramaniam *et al.*, *J. Biol. Chem.* **266**, 24613 (1991)] was obtained from J. Robbins, University of Cincinnati. The expression vector contained the 5.5-kb murine α-MHC promoter with 5′ noncoding exons upstream of the Sal I site into which a polymerase chain reaction (PCR)–amplified cDNA encoding rat cardiac TnI truncated at Lys^{193} was cloned. Downstream to the TnI insert is an approximately 600–base pair region of the human growth hormone (hGH) gene containing a polyadenylation signal. Sequencing confirmed the fidelity of the TnI fragment and its orientation in the vector. The MHC–TnI_{1-193}–hGH cassette was excised from the vector by Not I digestion, purified, and injected into pronuclear embryos (C57BL/6 × A/J). Founders were identified by Southern blotting and in subsequent generations by PCR analysis. Three founders were bred to C57BL/6 nontransgenic mates.
6. Immunoblotting was as described [N. M. Hunkeler, J. Kullman, A. M. Murphy, *Circ. Res.* **69**, 1409 (1991) (*3*, *4*)], except that the cardiac muscle was homogenized in 100 mM potassium phosphate buffer (pH 7.8) with 1 mM dithiothreitol, 2 mM EDTA, 1% Triton with protease inhibitors, then pelleted and resuspended in sample buffer.
7. Transgenic mice had a higher incidence of sudden death with anesthesia.
8. Mice were sedated with methoxyflurane and placed on a warming pad. Echocardiograms were obtained with a 15-MHz vascular probe with a standoff from the chest using an Acuson ultrasound machine.
9. RNA was prepared using Trizol reagent (GIBCO). Dot blots were prepared and hybridized with end-labeled transcript-specific oligonucleotide probes as described [W. K. Jones *et al.*, *J. Clin. Invest.* **98**, 1906 (1996); A. Sanbe *et al.*, *J. Biol. Chem.* **274**, 21085 (1999)], and expression was quantified using a Packard phosphorimager. Relative expression was normalized to expression of glyceraldehyde 3-phosphate dehydrogenase. The results from three nontransgenic mice were compared by *t*-test to the results from nine transgenic mice.
10. Mice were euthanized by deep methoxyflurane anesthesia. Tissues were fixed in formalin (Sigma), and paraffin-embedded sections were stained with hematoxylin and eosin, and with Masson's trichrome. A cardiac pathologist blinded to the identity of the mice reviewed multiple sections from base to apex of hearts from three nontransgenic and three transgenic mice. No differences in cellular histology or fibrosis could be distinguished.
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20. Informed consent was obtained after the nature and possible consequences of the studies were explained to patients undergoing coronary artery bypass surgery for ischemic disease. Cardiac ventricular muscle samples (50 to 100 μg) from noninfarcted areas were obtained after institution of cardiopulmonary bypass flow but before aortic cross-clamping and 10 min after removal of the aortic cross clamp and were immediately snap frozen in liquid nitrogen. The muscle was homogenized in 6 M urea in the presence of protease inhibitors and electrophoresed in SDS-polyacrylamide with 3 M urea, and immunoblots were prepared and labeled as in (*4*).
21. J. L. McDonough, G. Ropchan, D. Atar, S. MacKenzie, J. E. Van Eyk, *Circulation* **100**, I-767 (1999) and unpublished data.
22. The three-state model is as described [S. S. Lehrer, *J. Muscle Res. Cell. Motil.* **15**, 232 (1994)] and is reviewed in R. J. Solaro and J. Van Eyk [*J. Mol. Cell. Cardiol.* **28**, 217 (1996)] and K. A. Palmiter and R. J. Solaro [*Basic Res. Cardiol.* **92**, 63 (1997)].
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26. Paradoxically, a COOH-terminal recombinant mutant lacking an additional six residues compared to the mouse construct increased the calcium sensitivity of the ATPase activity of cardiac myofibrils (*24*). However, recombinant COOH-terminal mutants of TnI have not been tested in force-generating preparations. Thus, it is possible that loss of COOH-terminal amino acids may uncouple force from ATPase activity.
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29. The care of the animals and participation of human subjects in this study was in accordance with institutional and IACUC (Institutional Animal Care and Use Committee) guidelines. We thank J. Robinson and L. Jones for technical assistance, R. Hruban for reviewing the cardiac pathology, G. Ropchan for obtaining the human samples, D. Judge and J. Weiss for assistance with the echocardiography, and M. J. Murphy for support. Supported by an American Heart Association grant-in-aid and NIH grant HL 63038 to A.M.M., NIH grant HL 44065 and the Michel Mirowski M.D. Professorship of Cardiology to E.M., grant KO1873/1-1 of the Deutsche Forschungsgemeinschaft to H.K., and a Heart and Stroke Foundation of Canada (T-3759) and Medical Research Council of Canada (MT-14375) to J.E.V.E., who is an Ontario Heart and Stroke Scholar. J.L.M. is a Heart and Stroke Foundation of Canada Research Trainee.

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Role of the Enteric Nervous System in the Fluid and Electrolyte Secretion of Rotavirus Diarrhea

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The mechanism underlying the intestinal fluid loss in rotavirus diarrhea, which often afflicts children in developing countries, is not known. One hypothesis is that the rotavirus evokes intestinal fluid and electrolyte secretion by activation of the nervous system in the intestinal wall, the enteric nervous system (ENS). Four different drugs that inhibit ENS functions were used to obtain experimental evidence for this hypothesis in mice in vitro and in vivo. The involvement of the ENS in rotavirus diarrhea indicates potential sites of action for drugs in the treatment of the disease.

Rotavirus is the major cause of infantile gastroenteritis worldwide and is associated with about 600,000 deaths every year, predominantly in developing countries (*1*). Although two decades of research have significantly increased our understanding of virus immunology and have led to the development of an oral vaccine, our knowledge of the mechanisms that induce rotavirus diarrhea, nausea, and vomiting remains limited.

Rotavirus infects the mature enterocytes in the mid and upper villous epithelium of the small intestine, ultimately leading to cell death and villus atrophy. A striking observation in both animals and humans is that only a few percent of the mature villus epithelial cells and no crypt cells seem to be infected (*2*–*5*). Fluid secretion is usually ascribed to an imbalance between the secretory crypt cells and the mature absorptive villous epithelium. The death of the villus cells leads to a repopulation of the epithelium with immature secretory type cells. Mechanisms proposed to explain the rotavirus-induced intestinal secretion of fluid and electrolytes include villous ischemia (*5*) and a toxin-like effect by a nonstructural virus protein, NSP4 (*6*).

A localized systemic response triggered by rotavirus-enterocyte interaction has been proposed previously (*4*). According to Ste-

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