Besides the core structure conserved in all troponin I isoforms, cardiac troponin I (cTnI) has an N-terminal extension that contains phosphorylation sites for protein kinase A under β-adrenergic regulation. A restricted cleavage of this N-terminal regulatory domain occurs in normal cardiac muscle and is up-regulated during hemodynamic adaptation (Z.-B. Yu, L.-F. Zhang, and J.-P. Jin (2001) J. Biol. Chem. 276, 15753–15760). In the present study, we developed transgenic mice over-expressing the N-terminal truncated cTnI (cTnI-ND) in the heart to examine its biochemical and physiological significance. Ca^{2+}-activated actomyosin ATPase activity showed that cTnI-ND myofibrils had lower affinity for Ca^{2+} than controls, similar to the effect of isoproterenol treatment. In vivo and isolated working heart experiments revealed that cTnI-ND hearts had a significantly faster rate of relaxation and lower left ventricular end diastolic pressure compared with controls. The higher baseline relaxation rate of cTnI-ND hearts was at a level similar to that of wild type mouse hearts under β-adrenergic stimulation. The decrease in cardiac output due to lowered preload was significantly smaller for cTnI-ND hearts compared with controls. These findings indicate that removal of the N-terminal extension of cTnI via restricted proteolysis enhances cardiac function by increasing the rate of myocardial relaxation and lowering left ventricular end diastolic pressure to facilitate ventricular filling, thus resulting in better utilization of the Frank-Starling mechanism.

Cardiac muscle contraction is powered by actomyosin ATPase that is regulated by Ca^{2+}-binding to the troponin complex (1, 2). The troponin complex consists of three subunits: tropomyosin (TnC; the Ca^{2+}-binding subunit), troponin T (TnT; the tropomyosin-binding subunit), and troponin I (TnI; the inhibitory subunit) (2, 3). In addition to the core structure conserved in all TnI isoforms, cardiac TnI (cTnI) has an ~30-amino acid N-terminal extension that is not present in fast and slow skeletal muscle TnIs. This N-terminal extension does not contain binding sites for other thin filament proteins (3, 4) but contains serine residues 23 and 24, which are protein kinase A (PKA) substrates. With β-adrenergic stimulation, phosphorylation of these serine residues facilitates myocardial relaxation by decreasing the affinity of TnC for Ca^{2+} (5, 6).

In a rat tail-hanging model simulating the effect of weightlessness on the cardiovascular system, an N-terminal truncated cTnI was found to be up-regulated in the heart after 3–4 weeks of simulated weightlessness (7). This truncated cTnI is produced by restricted proteolysis, which removes the N-terminal amino acids 1–30. The restricted cleavage of cTnI selectively deletes the cardiac specific N-terminal extension, including the regulatory serine residues, but leaves the core structure intact (7).

It has been established that long term exposure to a weightless environment results in decreased cardiac function (8–10). A number of cardiovascular adaptations to weightlessness in microgravity and simulated microgravity occur in response to redistribution of body fluids to the head and neck region (11–16). In long term exposure to microgravity and simulated microgravity, this fluid redistribution results in increased renal discharge of Na^{+} and water to reduce circulatory blood volume and central venous pressure. According to the Frank-Starling relationship, cardiac function decreases as a result of this decreased preload. It is, therefore, important to investigate whether proteolytic removal of the cTnI N-terminal domain has a negative impact on myocardial contractility or whether it is a compensatory response to the decrease in cardiac preload. Characterization of the post-translational mechanisms in myocardial adaptation to long term exposure to microgravity will provide a better understanding of myocardial dysfunction in astronauts as well as in bedridden patients in which similar cardiovascular changes occur (10). The N terminus-truncated cTnI is present in normal hearts of all species examined (7), indicating that proteolytic removal of the N-terminal extension of cTnI, including the PKA substrate sites, may be a novel mechanism to regulate cardiac function under physiological and stress conditions. Understanding the functional effect of this structural modification of cTnI will increase our knowledge of mechanisms regulating myocardial contraction and may also identify a molecular target for potential therapeutic enhancement of cardiac function.

To develop an integrated experimental system for characterizing the effect of removing the N-terminal extension of cTnI on
cardiac function, we developed transgenic mice with postnatal overexpression of N terminal-truncated cTnI that lacks amino acids 1–28 (cTnI-N-D) for biochemical and physiological characterization. cTnI-N-D cardiac myofibrils showed a lower affinity for Ca\(^{2+}\) than controls in actomyosin ATPase assay, similar to the effect of isoproterenol treatment. cTnI-N-D hearts had a significantly faster rate of relaxation and lower left ventricular end diastolic pressure (LVEDP) compared with controls. The higher base-line relaxation rate of cTnI-N-D hearts was similar to that of wild type (WT) mouse hearts under \(\beta\)-adrenergic stimulation. The decrease in cardiac output due to lowered preload was significantly smaller for cTnI-N-D hearts compared with controls. These findings indicate that removal of the N-terminal extension of cTnI via restricted proteolysis enhances cardiac function by increasing the rate of myocardial relaxation and lowering LVEDP in order to facilitate ventricular filling for a better utilization of the Frank-Starling mechanism.

**MATERIALS AND METHODS**

**Construction of a cDNA Template Encoding cTnI-N-D**—To investigate the effect of removing the N-terminal domain of cTnI on cardiac muscle function, we engineered cDNA encoding mouse cTnI with a deletion of amino acids 1–28, thus mimicking the naturally occurring proteolytic truncation previously observed (1). cDNA encoding intact mouse cTnI was cloned by reverse transcription-coupled PCR. Total RNA was extracted from ventricular muscle of an adult 129Sv/d mouse using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The upstream (forward) and downstream (reverse) oligonucleotide primers were synthesized according to the published mouse cTnI cDNA sequence (17) corresponding to the regions around the translation initiation and termination codons, respectively. The first strand mouse cTnI cDNA was synthesized from the total RNA preparation using avian myeloblastosis virus reverse transcriptase. Double-stranded cDNA including the entire coding region was then amplified by PCR, cloned into the pAED4 plasmid as described previously (18). Full-length mouse cTnI cDNA, including the 3′-untranslated regions and the poly(A) tail, was then reconstituted by pasting a restriction enzyme fragment, cloned by PCR from a unidirectional mouse cardiac cDNA λ-ZAPII phage library (19) using a TnI-specific forward primer paired with T7 primer in the downstream flanking vector sequence. The mouse cTnI cDNA was sequenced and verified by protein expression in *Escherichia coli* and Western blot identification using an anti-TnI monoclonal antibody (mAb) (TnI-1) (20).

To construct a cDNA encoding the N terminus-truncated cTnI, an oligonucleotide primer was synthesized to create a translation initiation codon before Ala\(^{29}\) in mouse cTnI (Fig. 1A). An Ndel restriction enzyme site was included in the primer sequence for subsequent cloning. PCR-amplified mutagenesis was carried out on the full-length mouse cTnI cDNA template in pAED4 plasmid using the N-terminal deletion primer versus a 3′-flanking primer in the vector sequences. The resulting cDNA construct was cloned into pAED4 plasmid (Fig. 1B) and sequenced to verify fidelity of the PCR and cloning procedures. The cDNA template encoding cTnI-N-D was further verified by protein expression in *E. coli* and Western blot identification using anti-TnI mAb TnI-1 as described above.

**Production of Transgenic Mouse Lines Overexpressing cTnI-N-D in Cardiac Muscle**—In order to overexpress cTnI-N-D in mouse cardiac muscle, we constructed a transgene using the cloned promoter of the mouse cardiac \(\alpha\)-myosin heavy chain (\(\alpha\)-MHC) gene (21) kindly provided by Dr. Jeffrey Robbins, University of Cincinnati) to direct a cardiac specific expression of the truncated cTnI cDNA in transgenic mice. Since the C57 N-terminal truncation is observed as a post-translational regulator of myocardial function, the \(\alpha\)-MHC promoter, which is up-regulated in mouse hearts after birth was used to avoid potential embryonic adaptations. The construction of the transgene is shown in Fig. 1C. The transgene DNA segment was cleaved from the recombinant plasmid by restriction enzyme digestion at flanking sites and isolated by agarose gel electrophoresis. After being recovered from the gel slice by electrophoresis elution and purified by the QIAfilter minicolumn (Qiagen, Chatsworth, CA), the linear transgene DNA fragment was used in the production of transgenic mice. Using fertilized eggs from C57 mice, the pronucleus injection and embryo reimplantation were performed at the Transgenic Core Facility at Case Western Reserve University School of Medicine.

For the screening of the transgenic genotypes, genomic DNA was purified from mouse tail snips by the proteinase K digestion method (22). Transgenic founders were identified by PCR using an \(\alpha\)-MHC promoter-specific primer versus a cTnI-specific primer (Fig. 1C). The transgenic founders were bred with wild-type C57 mice, and the progenies were screened by PCR as above. Positive F1 progenies from each transgenic founder line were verified for the expression of cTnI-N-D in the cardiac muscle by TnI-1 mAb Western blot analysis. F2 generation with clear segregation of the transgene allele was used in this study for the characterization of cardiac function.

All mice were provided food and water *ad libitum* and placed on a 12/12-h light-dark cycle, with the light cycle occurring during the daytime. All animal procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and were conducted in accordance with the Guiding Principles in the Care and Use of Animals, as approved by the Council of the American Physiological Society.

**Verification of Cardiac Specific Expression and Myofilament Incorporation of cTnI-N-D**—Multiple types of somatic tissue were obtained from adult transgenic mice and homogenized in SDS-PAGE sample buffer containing 2% SDS. The protein extracts were analyzed by SDS-PAGE and Western blot using anti-TnI mAb TnI-1 as described previously to verify cardiac specific expression of cTnI-N-D.

To examine incorporation of cTnI-N-D into cardiac myofibrils, ventricular myofibrils were isolated as described previously (22) and examined by Western blotting as above. To evaluate the effect of cTnI-N-D on related myofilament proteins, the expression and myofilament incorporation of cardiac TnT in the transgenic mouse ventricular mus-
Preparation of Cardiac Myofibrils and Actomyosin ATPase Assay—
Myofibrils were isolated from wild type cardiac muscle using a modification of the method described previously (22). Briefly, hearts were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally). Hearts removed from transgenic or control mice were perfused in a retrograde manner, either with normal saline or with saline containing 20 mM isoproterenol. Cardiac myofibrils were prepared according to the method described by Solaro et al. (23) with modifications. These include the use of NaF (50 mM), EGTA (2.5 mM), and K$_3$PO$_4$ as phosphate inhibitors and phenylmethylsulfonyl fluoride (0.2 mM) as a protease inhibitor (24). The ventricular muscle was pulverized in a food blender for 10 s in 50 ml of buffer containing 0.5% (v/v) Triton X-100, 60 mM KCl, 30 mM imidazole, pH 7.0, 2 mM MgCl$_2$, and 1 mM dithiothreitol, incubated for 5 min, and centrifuged at 2,500 × g for 2 min. Pellets were washed three times in buffer containing 60 mM KCl, 30 mM imidazole, pH 7.0, 2 mM MgCl$_2$, and 1 mM dithiothreitol. Adenosine triphosphate (ATP) and Ca$^{2+}$ sensitivity of the transgenic and wild type mouse cardiac myofibrils under constant ionic strength, using the stability constants compiled by Fabiato (25), and were performed at pH 7.0 with 50 mM imidazole, 50 mM KCl, and 2 mM MgATP at 37 °C. Inorganic phosphate release was measured as the amount of heteropolyoxymetlenylenzene (26) using an automated microtiter plate reader (Bio-Rad Benchmark) for optical density at 660 nm. The data are expressed as mean ± S.D.

Measurement of Left Ventricular Pressure Development in Vivo—Intraventricular pressure measurements were obtained in vivo using a Millar catheter (model SPR 671; Millar Instruments, Houston, TX) as per the method of Kass et al. (28) with some modification. Briefly, mice were anesthetized with sodium pentobarbital as above, and a small abdominal incision was made to gain access to the pericardium without disturbing the integrity of the thoracic cavity. An 18-gauge sheath was introduced into the left ventricle through a catheter, and the heart was switched to working mode. The hearts were perfused with Krebs-Henseleit bicarbonate buffer aerated with 95% O$_2$, 5% CO$_2$ at 37 °C (pH 7.4). The buffer contents were as follows: 118 mM NaCl, 4.7 mM KCl, 2.25 mM CaCl$_2$, 2.25 mM MgSO$_4$·7H$_2$O, 1.2 mM KH$_2$PO$_4$, 0.32 mM EGTA, 25 mM NaHCO$_3$, and 11 mM D-glucose (29). Function of the isolated working mouse heart was determined from the regression equation, where heart weight was plotted against stroke volume. cTnI-ND transgenic mouse lines were obtained with successful segregation of the transgene. All functional experiments were performed in a blinded manner. cTnI-ND expression levels in all hearts used in functional studies were confirmed by Western blot using mAb TnI-1 as described above. Densitometric quantification of SDS-gel bands and Western blots was carried out using NIH Image 1.61 software. Based on an average heart weight of 0.130 g and an average heart rate of 467 beats/min, an estimated stroke volume of 179 μl/g of heart weight was predicted, based on data obtained from 60 isolated working wild type mouse hearts under the afterload of 55 mm Hg and preload of 10 mm Hg. This value was determined from the regression equation, $y = 32.4x + 4.2$, $r = 0.83$, where heart weight was plotted against stroke volume.

Hemodynamic values (stroke volume, relative ±dP/dt, intrinsic heart rate, pressure-time integrals, systolic and diastolic durations, and peak/relaxation times) were initially tested for homogeneity of variance with a Levene test to determine whether a value was parametric or nonparametric (44). Statistical significance was determined using either analysis of variance (for parametric parameters) or a Kruskal-Wallis analysis of variance (for nonparametric parameters). If a significant difference was identified by analysis of variance, multiple comparisons for parametric data were examined with a Scheffe post hoc test. For nonparametric data, if a significant difference was found with a Kruskal-Wallis analysis of variance, multiple comparisons were performed using a Tamhane post hoc test. All statistical tests were performed with the use of SPSS statistical software. The 5% level of confidence was arbitrarily used for assigning statistically significant differences, and all data are presented as mean ± S.D.

**RESULTS**

cTnI-ND Transgenic Mouse Lines—Four original transgenic mouse lines were obtained with successful segregation of the transgene allele. Western blots using anti-cTnI mAb TnI-1 showed cardiac specific expression of the exogenous cTnI-ND (Fig. 2A). Similar to that previously observed in transgenic mouse lines (23), overexpression of cTnI-ND resulted in a significant replacement of endogenous cTnI. Densitometry of Western blots showed that in wild type mouse hearts, cTnI-ND produced by endogenous proteolysis is within a range of up to 10% of the amount of total cTnI (intact plus truncated), similar to that found in the rat hearts (7). From the four transgenic mouse lines, two distinct groups of transgenic mouse hearts were identified with different amounts of cTnI-ND in the ventricular muscle relative to the total cTnI (Fig. 2B). Transgenic mice expressing cTnI-ND at 16–30% of total cTnI were classified as

| Heart rate (beats/min) | WT (n = 6) | H-cTnI-ND (n = 6) |
|------------------------|-----------|------------------|
| 549 ± 11               | 554 ± 12  |
| LVP (mm Hg)            | 116.4 ± 3 | 101.2 ± 2*       |
| LVEDP (mm Hg)          | 5.88 ± 1.7| 1.61 ± 0.51*     |
| −dP/dt (mm Hg s⁻¹)     | 4899 ± 635| 5321 ± 344       |
| +dP/dt (mm Hg s⁻¹)     | 3387 ± 544| 5283 ± 250*      |

* Significantly different from the WT values (p < 0.01).

LVP, left ventricular pressure.
N-terminal Truncation of Cardiac Troponin I

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of hypertrophy (no significant differences in heart weight/body weight ratio (Table II) or diameter of cardiac myocytes as compared with wild type controls) and no evidence of anatomical or histological abnormality were found in the transgenic mouse hearts (data not shown).

N-terminal Truncation of cTnI Decreases Ca\(^{2+}\) Sensitivity of Myofibrin Actomyosin ATPase—The maximum Ca\(^{2+}\)-activated actomyosin ATPase rate was similar in cTnI-ND and WT mouse cardiac myofibrils (417 ± 48 and 422 ± 73 nmol of P\(_i\)/mg of protein/min, respectively). Compared with myofibrils isolated from WT mouse hearts, H-cTnI-ND myofibrils were less sensitive to Ca\(^{2+}\) in an actomyosin ATPase assay (Fig. 3). Shown by the rightward shift of the ATPase-pCa curve, H-cTnI-ND myofibrils had significantly lower pCa\(_{50}\) values compared with that of WT (5.88 ± 0.03 versus 6.15 ± 0.05, p < 0.01). This decreased Ca\(^{2+}\) sensitivity is comparable with that achieved in WT mouse controls in response to β-adrenergic stimulation. In other words, the base-line pCa\(_{50}\) value for non-isoproterenol-treated H-cTnI-ND myofibrils was similar to myofibrils from isoproterenol-perfused WT hearts (5.88 ± 0.03 versus 5.91 ± 0.05). In contrast, isoproterenol perfusion of the transgenic mouse hearts did not further decrease Ca\(^{2+}\) sensitivity in H-cTnI-ND myofibrils, consistent with the absence of PKA phosphorylation sites. The results also indicate no additive effect of β-adrenergic stimulation and N-terminal truncation of cTnI, suggesting a common target for the two regulatory mechanisms.

Base-line in Vivo Parameters of H-cTnI-ND and WT Mouse Hearts—Table I summarizes the in vivo measurements obtained for WT and H-cTnI-ND mouse hearts. The heart rate was similar between WT and H-cTnI-ND mice. A small but statistically significant lower (by 13%) left ventricular pressure was found in H-cTnI-ND mice compared with the WT control. In contrast, LVEDP values were much lower (by 73%) in H-cTnI-ND than the control. Whereas no significant difference in the velocity of ventricular contraction (+dP/dt) was found between the two groups, H-cTnI-ND mouse hearts exhibited significantly faster rates of relaxation (−dP/dt) than that of WT control.

Base-line Working Parameters of Isolated Transgenic Mouse Hearts—The measurements are summarized in Table II. Intrinsic heart rates of 460–470 beats/min were obtained for isolated working hearts in all experimental groups. This nearly in vivo heart rate in the absence of artificial pacing demonstrates viability of the working heart preparation. At 10 mm Hg preload, stroke volumes of H-cTnI-ND hearts were significantly greater than WT and INT-cTnI-ND groups. Similar to in vivo measurements (Table 1), +dP/dt values measured during working heart experiments were not significantly different among WT, INT-cTnI-ND, and H-cTnI-ND groups. Consistently, the time to peak pressure is similar among the three groups.

A comparison of −dP/dt showed that the H-cTnI-ND and INT-cTnI-ND group had significantly faster rates of relaxation than WT hearts. As derived from data shown in Table II, H-cTnI-ND and INT-cTnI-ND hearts relaxed 24.7 and 14.5% faster than WT hearts, respectively. As a result of this increase in −dP/dt, the H-cTnI-ND hearts uniquely demonstrated a pattern of faster relaxation than contraction (Table II). Compared with the relaxation time for WT controls, H-cTnI-ND hearts showed shorter durations throughout the entire course of relaxation (RT\(_{10}\), RT\(_{20}\), and RT\(_{50}\)), whereas INT-cTnI-ND hearts showed a shorter RT\(_{10}\) (Table II).

As a consequence of faster relaxation, the duration of systole measured as the time interval between the beginning of pressure generation and the rapid decrease of pressure marked by the closure of the aortic valve was significantly shorter for
50 nM isoproterenol perfusions produced significant increases versus respectively, base-line value of H-cTnI-ND hearts (2087 WT controls (Table II), 10 nM isoproterenol produced the ex-
cardiac myofibrils (417 48 and 422 73 nmol of P/mg of protein/min for the base line and 421 43 and 418 57 nmol of P/mg of protein/ min with isoproterenol treatment, respectively).

H-cTnI-ND and INT-cTnI-ND hearts than the WT control. Since the heart rates were similar in all groups, this abbrevi-
atuated duration of systole resulted in a longer duration of diastole for the H-cTnI-ND hearts. The faster relaxation and longer
diastole is consistent with the lower values of LVEDP measured in vivo (Table I).

Unique Effects of Isoproterenol Treatment on cTnI-ND Hearts—Fig. 4A shows the response of H-cTnI-ND hearts to 10, 30, and 50 nM isoproterenol perfusion during working heart experiments. As expected, the velocity of contraction (+dP/dt) increased in all three groups in response to 10 nM isoproterenol. 30 and 50 nM isoproterenol perfusion data showed a plateau of this effect.

Whereas cTnI-ND hearts had higher base-line −dP/dt than WT controls (Table II), 10 nM isoproterenol produced the ex-
pected positive effect on WT hearts (Fig. 4B). However, the same treatment had less effect on INT-cTnI-ND hearts and no effect on H-cTnI-ND hearts. It is interesting to note that the increased −dP/dt values in WT and INT-cTnI-ND hearts produced by 10 nM isoproterenol treatment are similar to the base-line value of H-cTnI-ND hearts (2087 ± 58 and 2040 ± 63, respectively, versus 2102 ± 196 mm Hg/s). Nonetheless, 30 and 50 nM isoproterenol perfusions produced significant increases in relaxation velocity in all three groups of hearts to reach similar absolute values (Fig. 4B). This result indicates a pre-
served β-adrenergic potential and precludes negative changes in the β-adrenergic system of cTnI-ND hearts.

Effects of cTnI-ND on Cardiac Function Responding to Changes in Preload—Fig. 5 illustrates the stroke volume produced by isolated WT and H-cTnI-ND working hearts at vari-
ous preloads. Increases in preload pressure resulted in increases in stroke volume in WT and H-cTnI-ND hearts, but

| TABLE II  
| Hemodynamic parameters of isolated working hearts |
|---------------------------------------------------|
| The measurements were done under a constant preload of 10 mm Hg. The data are shown as mean ± S.D. |
| WT (n = 16) | INT-cTnI-ND (n = 7) | H-cTnI-ND (n = 11) |
| Heart weight/body weight (mg/g) | 4.30 ± 0.36 (×10⁻²) | 4.26 ± 0.18 (×10⁻²) | 4.24 ± 0.19 (×10⁻²) |
| Heart rate (beats/min) | 461 ± 13 | 469 ± 11 | 471 ± 5 |
| Stroke volume (μL of tissue⁻¹) | 134 ± 7.3 | 128 ± 8.5 | 159 ± 4.9⁠a |
| +dP/dt (mm Hg s⁻¹) | 1968 ± 204 | 1994 ± 243 | 1964 ± 200 |
| −dP/dt (mm Hg s⁻¹) | 1866 ± 114 | 1930 ± 268⁠a | 2102 ± 196⁠b |
| Duration of systole (ms) | 47.8 ± 1.79 | 43.0 ± 1.1⁠a | 40.4 ± 1.1⁠a |
| Duration of diastole (ms) | 78.6 ± 1.52 | 83.33 ± 1.03 | 84.6 ± 1.95⁠a |
| Time to peak pressure (ms) | 38.6 ± 2.88 | 37.3 ± 2.67 | 38.4 ± 1.74 |
| RT₅₀ (ms) | 9.9 ± 3.10 | 9.0 ± 0.7⁠a | 7.11 ± 0.5⁠b |
| RT₇₅ (ms) | 33.6 ± 4.38 | 34.3 ± 2.51 | 29.8 ± 2.1⁠b |
| STI (mm Hg/s) | 52.1 ± 5.25 | 52.6 ± 3.09 | 47.3 ± 2.6⁠b |
| DTI (mm Hg/s) | 2.64 ± 0.112 | 2.43 ± 0.044⁠a | 2.25 ± 0.045⁠a |
| Stroke work (ml/mm Hg of tissue) | 3.45 ± 0.089 | 3.79 ± 0.08⁠a | 4.11 ± 0.07⁠b |

⁠a Different from the WT group (p < 0.01). 
⁠b Different from the INT-cTnI-ND group (p < 0.05).

FIG. 3. Removal of the N-terminal domain of cTnI decreases the Ca²⁺ sensitivity of actomyosin ATPase. Normalized Ca²⁺-dependent actomyosin ATPase activation curves for isolated myofibrils were obtained from WT and H-cTnI-ND hearts with or without isoproterenol treatment. The results demonstrate that H-cTnI-ND myofibrils are less sensitive to Ca²⁺ activation, similar to WT hearts upon β-adrenergic stimulation. In contrast, isoproterenol treatment did not produce any further decrease in Ca²⁺ sensitivity in H-cTnI-ND myofibrils. The maximum ATPase rate was similar in cTnI-ND and WT mouse cardiac myofibrils (417 ± 48 and 422 ± 73 nmol of P/mg of protein/min for the base line and 421 ± 43 and 418 ± 57 nmol of P/mg of protein/ min with isoproterenol treatment, respectively).

Differences in Preload—Fig. 5 illustrates the stroke volume pro-
duction of working hearts perfused with various concentrations of isoproterenol (iso). Data are shown as mean ± S.D. * significantly different from WT base-line value (p < 0.01); † significantly different from INT-cTnI-ND base-line value (p < 0.05); ‡ significantly different from 10 nM iso value of the same group (p < 0.05).

FIG. 4. Effects of isoproterenol treatment on the velocity of ventricular contraction and relaxation. The bar graphs represent the average values for +dP/dt (A) and −dP/dt (B) measured from WT (n = 16), INT-cTnI-ND (n = 7), and H-cTnI-ND (n = 11) mouse working hearts perfused with various concentrations of isoproterenol (iso).
H-cTnI-ND hearts produced higher stroke volume than WT heart controls at all four preload pressures tested, with a bigger ratio at 3.5 mm Hg. The facilitated ventricular relaxation of H-cTnI-ND hearts shown by the faster ratio at 3.5 mm Hg. The increased end-diastolic volume will explain the higher stroke volume produced by H-cTnI-ND hearts.

Whereas the increased end-diastolic volume due to better ventricular relaxation will result in higher contractility by the Frank-Starling mechanism, the similar maximum myofilament actomyosin ATPase rate, +dP/dt, and systolic pressure in the WT and cTnI-ND hearts indicate that the higher stroke volume produced by cTnI-ND hearts is unlikely to be due to an intrinsic increase of contractility. In agreement with the hypothesis that cTnI-ND hearts have an enhanced ventricular relaxation and would be more tolerant to decreases in preload pressure, the reduction of cardiac output when preload pressure was lowered from the standard experimental condition of 10 to 3.5 mm Hg in isolated hearts was less severe in H-cTnI-ND mouse hearts (Fig. 5; the output was 1.86-fold more than that of WT hearts).

Overall Effect of cTnI-ND on Cardiac Performance—Integrating the pressure wave for the duration of systole and diastole yielded values for STI and DTI, respectively (38). The cTnI-ND hearts showed significantly lower STI than WT controls (Table II). Conversely, the largest DTI value was found for the H-cTnI-ND group, 19% greater than the WT control (Table II). DTI of INT-cTnI-ND was 10% greater than the WT control (Table II).

Fig. 6A illustrates a regression of STI against stroke work. The WT heart data showed an expected positive correlation of STI with stroke work (r = 0.92). An increase in STI of 1.0 mm Hg/s corresponds to an increase in stroke work of 2.49 ml/mm Hg/g of heart tissue. Interestingly, the H-cTnI-ND group data deviated from this regression and clustered at the lower end of the STI scale but at the higher end of the stroke work scale. Extrapolating from the corresponding WT regression data, the H-cTnI-ND data reflect a production of 69% more stroke work at a lower STI value. Indicating a dose response to the level of N terminus truncated cTnI, the INT-cTnI-ND data distributed between the H-cTnI-ND and WT groups and reflect 21% more stroke work relative to the WT regression curve.

On the other hand, WT, INT-cTnI-ND and H-cTnI-ND hearts showed similar positive DTI versus stroke work relationships (r = 0.90, 0.88, and 0.91, respectively). Fig. 6B illustrates a combined regression curve of DTI versus stroke work for all three groups (r = 0.89), where a 1.0 mm Hg/s increase in DTI corresponds to a 3.29 ml/mm Hg/g heart tissue increase in stroke work.

**DISCUSSION**

**Regulation of cTnI Function by Restricted Proteolysis—**Post-translational modification forms an important regulation of cTnI function. PKA-catalyzed phosphorylation of Ser<sup>23</sup> and Ser<sup>24</sup> in the N-terminal domain of cTnI has been extensively investigated for roles in β-adrenergic response. Whereas proteolytic degradation of cTnI at the C terminus was found to result in myocardial dysfunction (46, 47), a physiological regulation of myocardial function by restricted cleavage of cTnI at the N terminus is a novel hypothesis. Selectively removing the N-terminal extension of cTnI retains the conserved core structure of TnI (Fig. 1A) and thus would not disrupt the formation of the troponin complex as shown in previous in vitro studies (48). Although this modification of cTnI structure was originally discovered in simulated microgravity, similar cTnI fragments are found at low levels in normal hearts of all species examined, indicating a much broader physiological relevance (7). This observation suggests a role of cTnI-ND in modulating
normal function of the heart as well as during myocardial adaptation to stress conditions. Whereas contractile force and velocity was reduced in cardiac muscle of tail suspension rats (7), it was unclear whether the decrease in contractility was caused by removal of the N-terminal domain of cTnI or the up-regulation of cTnI-ND was a compensatory adaptation. To test the hypothesis that the proteolytic N-terminal truncation of cTnI is an intrinsic post-translational mechanism to modulate thin filament-based Ca^{2+} regulation of cardiac muscle, the present study provides plausible evidence that restricted deletion of the N-terminal domain of cTnI facilitates relaxation of cardiac muscle and enhances cardiac function.

The cTnI-ND transgenic mice provide an integrated experimental system to study myocardial function under physiological conditions. In addition to biochemical activity at the myofibrillar level and in vivo evaluation of cardiac function, the isolated working heart allows detailed measurements of ventricular function under various hemodynamic conditions and pharmacological stimulation without the complexities of systemic neurohumoral regulation (29–31). The apparently normal cardiac function of the transgenic mice, with up to 82% of cTnI replaced by cTnI-ND supports the hypothesis that the selective deletion of cTnI N-terminal domain is not destructive. In fact, our data show that removal of the N-terminal extension of cTnI enhances myocardial relaxation, indicating a positive regulatory mechanism of cardiac function. Therefore, the increased levels of proteolytic removal of cTnI N-terminal domain during long term exposure to simulated microgravity may be an adaptive mechanism to compensate for cardiac function when circulatory blood volume is reduced (7).

**Beneficial Effect of Increased Myocardial Relaxation on Heart Function—** Ventricular filling depends upon the venous return and the relaxation of the ventricular muscle. A logical consequence of the increased rate of myocardial relaxation and time available for ventricular filling (the abbreviated duration of systole and longer duration of diastole) is a larger end-diastolic volume with an effect on the length-tension relationship of the ventricular muscle (37–39). An increase in ventricular relaxation will result in a lower end-diastolic pressure and more ventricular filling. Indeed, Fig. 5 shows that cTnI-ND hearts produced higher output than the control. The ratio is significantly bigger at 3.5 mm Hg preload pressure versus the higher preload pressures. This finding suggests that cTnI-ND hearts have a greater ventricular relaxation compared with the WT hearts, which would allow for better utilization of the Frank-Starling mechanism. This Frank-Starling mechanism-based beneficial effect can be seen in Fig. 6B as the positive correlation between DTI and stroke work in cTnI-ND as well as in WT hearts, in which H-cTnI-ND heart data clustered at the higher end of the stroke work scales. This hypothesis is consistent with the potentially compensatory up-regulation of cTnI-ND in simulated long term microgravity (7). By having a faster ventricular relaxation and longer diastolic duration, the cTnI-ND hearts would allow for better filling of the ventricle especially when central venous pressure (preload) is lowered in adaptation to microgravity (15, 49). This observation indicates that cTnI N-terminal truncation is an attractive mechanism to improve heart function by enhancing the diastolic function of cardiac muscle.

Nonetheless, the preload and afterload in vivo depend on the characteristics of both the heart and the vascular system. Guyton et al. (50) developed the concept that the control of cardiac output in vivo is a complex interplay of cardiac function and vascular function. The cardiac output varies directly with central venous pressure, whereas the central venous pressure varies inversely with cardiac output. Although in vivo cardiac output was not measured in the present study, an increase in cardiac output in the working heart experiments supports the hypothesis that the reduced LVEDP of cTnI-ND mice (Table I) would result in better filling of the ventricle and increased cardiac output in vivo. Nonetheless, the potential contribution of secondary changes in the vascular system to the cTnI-ND-originated cardiac regulation remains to be investigated.

Neely et al. (31) demonstrated that the STI positively correlates with myocardial oxygen demand (32–34). Similarly, we showed that STI values correlate positively with stroke work in control mouse hearts (Fig. 6A). In contrast, the significant deviation from this curve by H-cTnI-ND hearts suggests that the transgenic mouse hearts can maintain outputs similar to WT hearts at a lower pressure-generating expense mainly due to abbreviated systolic duration (Table II). The INT-cTnI-ND group data are between that of WT and H-cTnI-ND groups, supporting a dose effect of N-terminal truncated cTnI.

Previous studies have established that the DTI/STI ratio correlates closely with the blood supply to the subendocardium (37–42). Because coronary perfusion occurs during diastole, a faster rate of relaxation would produce a longer duration of diastole, yielding deeper perfusion from the coronary arteries. Although overall coronary flows of the experimental groups were similar and proportional to heart weight/body weight ratios, the higher DTI/STI ratios of cTnI-ND hearts may also be beneficial by allowing better subendocardial perfusion.

**Potential Mechanism for cTnI-ND to Increase the Rate of Cardiac Muscle Relaxation—** A key finding of our study was that deletion of the N-terminal 30 amino acids of cTnI increased $-dP/dt$ in transgenic mouse hearts in vivo (Table I) and in isolated working preparations (Table II). The increased velocity of relaxation provides the basis of increased duration of diastole (Table II) and significantly decreased LVEDP (Table I) to enhance cardiac function. The N-terminal region of cTnI contains the PKA phosphorylation sites, serine 23 and serine 24 (51). Upon β-adrenergic stimulation, PKA-catalyzed phosphorylation of these sites enhances relaxation by decreasing the affinity of TnC for Ca^{2+} (52). Consistently, we found increased $-dP/dt$ in response to 10 nM isoproterenol perfusion in WT mouse hearts. However, the same level of β-adrenergic stimulation did not produce an increase in $-dP/dt$ in H-cTnI-ND hearts above the high baseline value (Fig. 4B). This unique phenomenon suggests that under baseline conditions, $-dP/dt$ for H-cTnI-ND hearts may already be at the same level achieved in WT hearts as a result of moderate β-adrenergic stimulation.

In agreement with the increased rate of myocardial relaxation, myofibrils isolated from cTnI-ND hearts demonstrated decreased Ca^{2+} sensitivity in actomyosin ATPase assays (Fig. 3). Consistent with previous studies (52), WT cardiac myofibrils showed a decrease in Ca^{2+} sensitivity upon isoproterenol treatment. However, cTnI-ND myofibrils did not show such a response. This observation is consistent with a recent study in which removal of the N-terminal extension of cTnI mimics the effect of PKA phosphorylation (53), proposing a hypothesis that proteolytic N-terminal truncation of cTnI may have similar effects on cardiac muscle relaxation as that produced by phosphorylation of serines 23 and 24 on cTnI during β-adrenergic stimulation.

On the other hand, higher levels of isoproterenol perfusions (30 and 50 nM) produced significant increases in relaxation velocity in the cTnI-ND hearts to a level similar to that produced in the WT hearts (Fig. 4B). This result indicates that the β-adrenergic potential of cTnI-ND hearts is preserved and β-adrenergic stimulation remains effective through other dominant mechanisms, such as increased sarcoplasmic reticulum...
uptake of Ca\textsuperscript{2+} via phospholamban phosphorylation (54).

Quantitative differences between H-cTnI-ND and INT-cTnI-ND hearts further suggest that the rate of relaxation is affected by the number of cTnI-ND incorporated into the myofilaments (Fig. 2D). The dose-dependent effect is indicated by the findings that INT-cTnI-ND hearts had relaxation rates, systolic durations, and STI and DTI values between values of WT and H-cTnI-ND hearts. Although INT-cTnI-ND hearts responded to β-adrenergic stimulation, the magnitude was smaller than observed in WT hearts. The quantitative effect for cTnI-ND to modulate cardiac muscle relaxation is suitable for a physiological regulatory mechanism during adaptation to functional demands.

Mammalian and avian embryonic cardiac muscles express slow skeletal muscle TnI that is replaced by cTnI during cardiac development (55, 56). This developmental switch of gene expression suggests that the N-terminal extension of cTnI, a structure absent from slow TnI, may play a unique role in the function of adult cardiac muscle. The fact that similar to phosphorylation within this region, removal of the N-terminal domain from cTnI in adult heart facilitates relaxation provides new evidence for its role in regulating myocardial diastolic function. The diminished response in −dP/dt of cTnI-ND hearts to β-adrenergic stimulation is similar to that observed in transgenic mouse muscles expressing slow TnI that also lacks the PKA phosphorylation sites (45, 52). However, the opposite effects of cTnI-ND and slow TnI on the Ca\textsuperscript{2+} sensitivity of cardiac muscle indicate functional differences due to the structural diversity in other regions of the TnI isoforms. For example, the functionally important C-terminal structure that is significantly different in cardiac and skeletal muscle TnI isoforms warrants further investigation.

It is worth noting that +dP/dt did not decrease in the cTnI-ND hearts. Therefore, myosin activity may be a dominant factor determining the maximum contractile velocity, whereas the relaxation velocity is dependent on the thin filament regulation. Since +dP/dt did not change but developed pressure was lower in the cTnI-ND heart in vivo (Table I), these hearts might be working against a lower afterload. Therefore, secondary changes in the vascular system may also be present in the transgenic mouse model and contribute to the physiological effects of overexpressing N terminus-truncated cTnI.

The functional effects of cTnI N-terminal truncation present a novel post-translational target for improving diastolic function of the heart. The increased β-adrenergoreceptor responsiveness in simulated microgravity indicates a role of the β-adrenergic system during cardiovascular adaptation (49). However, β-adrenergic stimulation has a wide range of biological consequences that limit clinical application. In contrast, removal of the N-terminal domain of cTnI by restricted cleavage selectively utilizes a beneficial effect of the β-adrenergic system. Whereas further research is needed to fully understand the mechanisms responsible for the modulation of cTnI function by N-terminal truncation, studies on mechanisms that control the restricted cleavage of the N-terminal extension of cTnI are under way in order to better understand thin filament adaptation in astronauts as well as in chronic bedridden and heart failure patients.

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