

Removal of the N-terminal Extension of Cardiac Troponin I as a Functional Compensation for Impaired Myocardial β -Adrenergic Signaling*

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Although β -adrenergic stimuli are essential for myocardial contractility, β -blockers have a proven beneficial effect on the treatment of heart failure, but the mechanism is not fully understood. The stimulatory G protein α -subunit ($G_s\alpha$) couples the β -adrenoreceptor to adenylyl cyclase and the intracellular cAMP response. In a mouse model of conditional $G_s\alpha$ deficiency in the cardiac muscle ($G_s\alpha$ -DF), we demonstrated heart failure phenotypes accompanied by increases in the level of a truncated cardiac troponin I (cTnI-ND) from restricted removal of the cTnI-specific N-terminal extension. To investigate the functional significance of the increase of cTnI-ND in $G_s\alpha$ -DF cardiac muscle, we generated double transgenic mice to overexpress cTnI-ND in $G_s\alpha$ -DF hearts. The overexpression of cTnI-ND in $G_s\alpha$ -DF failing hearts increased relaxation velocity and left ventricular end diastolic volume to produce higher left ventricle maximum pressure and stroke volume. Supporting the hypothesis that up-regulation of cTnI-ND is a compensatory rather than a destructive myocardial response to impaired β -adrenergic signaling, the aberrant expression of β -myosin heavy chain in adult $G_s\alpha$ -DF but not control mouse hearts was reversed by cTnI overexpression. These data indicate that the up-regulation of cTnI-ND may partially compensate for the cardiac inefficiency in impaired β -adrenergic signaling.

The β -adrenoreceptor (β -AR)³ signaling pathway plays an important role in the regulation of heart function (1–3). Activation of β -AR by catecholamines stimulates adenylyl cyclase and cAMP production via coupling to stimulatory G-protein (G_s), which in turn leads to activation of cAMP-dependent protein kinase (PKA) and phosphorylation of a multitude of intra-

cellular substrates in the Ca^{2+} handling system (sarcolemmal L-type Ca^{2+} channels (4), the ryanodine receptor (5), and phospholamban in sarcoplasmic reticulum (6), and myofilament proteins (cardiac troponin I (cTnI) (7) and myosin-binding protein C (8)).

In heart failure patients, the level of plasma norepinephrine is enhanced, resulting from activation of sympathetic nervous system, which induces chronic stimulation of cardiac β -adrenergic receptors (9). Although acute β -stimulation enhances cardiac function to adapt to systemic needs, chronic stimulation of the β_1 -adrenergic receptor is detrimental and contributes to cardiomyocyte hypertrophy, cell death, and progression of heart failure (10–12). Transgenic mice overexpressing β_1 -adrenoreceptor (13) or the G_s subunit α ($G_s\alpha$) (14) in the heart developed heart failure with increased level of apoptosis and fibrotic degeneration similar to that observed in dilated cardiomyopathy in humans. Overexpression of the catalytic subunit of PKA also produced cardiomyocyte hypertrophy, fibrosis, and a progressive decline in cardiac function, resulting in heart failure (15). On the other hand, failing hearts respond to the chronically elevated norepinephrine concentrations by desensitizing their response to β -adrenergic stimulation (11, 16).

Cardiac TnI is the inhibitory subunit of the troponin complex and plays an essential role in Ca^{2+} regulation of cardiac muscle contraction. Cardiac TnI is a substrate of PKA and is phosphorylated upon β -adrenergic stimulation (7). The PKA phosphorylation sites are two adjacent serine residues, Ser²³/Ser²⁴ (rat/mouse residue numbers), located in the cardiac specific N-terminal extension of cTnI. β -Adrenergic stimulated phosphorylation of cTnI Ser²³/Ser²⁴ reduces myofilament Ca^{2+} sensitivity (7, 17) and increases the rate of cardiac muscle relaxation (18–21). PKA-dependent cTnI phosphorylation is decreased in heart failure (22, 23).

A restricted N-terminal truncation of cTnI occurs at low levels in normal heart and increases in adaptation to hemodynamic changes in the tail suspension rat model of simulated microgravity (24). Peptide sequencing showed that this post-translational modification preserves the core structure of TnI but selectively removes the cTnI-specific N-terminal extension, including the PKA phosphorylation sites Ser²³ and Ser²⁴. Transgenic mouse hearts overexpressing the N-terminal truncated cTnI (cTnI-ND) demonstrated increased myocardial relaxation and improved ventricular filling for a better utiliza-

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³ The abbreviations used are: β -AR, β -adrenoreceptor; $G_s\alpha$, G protein α subunit; $G_s\alpha$ -DF, stimulatory G protein α subunit deficiency; cTnI, cardiac troponin I; cTnI-ND, N-terminal truncated cardiac troponin I; MHC, myosin heavy chain; PKA, protein kinase A; P-V, pressure-volume; MCK, muscle creatine kinase.

tion of the Frank-Starling mechanism, mimicking the effect of PKA phosphorylation (25). These results suggest that the β -adrenergic signaling pathway affects cTnI function by both phosphorylation and proteolytic modulation to regulate myocardial contraction.

In the present study, we showed that impaired β -adrenergic signaling resulting from myocardial $G_s\alpha$ deficiency ($G_s\alpha$ -DF) leads to impaired cardiac function accompanied by an up-regulation of cTnI-ND. Overexpression of cTnI-ND in $G_s\alpha$ -DF hearts increased relaxation velocity and left ventricular end diastolic volume to produce higher left ventricle maximum pressure and stroke volume. Supporting the hypothesis that the up-regulation of cTnI-ND is a compensatory rather than a destructive myocardial response to impaired β -adrenergic signaling, cTnI-ND overexpression reversed the aberrant expression of β -myosin heavy chain (β -MHC) in $G_s\alpha$ -DF hearts. These data indicate that up-regulation of cTnI-ND may partially compensate for the cardiac inefficiency in impaired β -adrenergic signaling, suggesting a potential therapeutic target for the treatment of heart failure.

EXPERIMENTAL PROCEDURES

Production of $G_s\alpha$ -DF in Mouse Cardiac Muscle—Mice with floxed $G_s\alpha$ exon 1 allele ($E1^f$) (26) were bred with the muscle creatine kinase (MCK)-*cre* mice (Taconic, Hudson, NY) to induce striated muscle-specific disruption of the $G_s\alpha$ gene (MCK-*cre*, $E1^{f/f}$; MG $_s$ KO). The $E1^f$ allele has no effect on $G_s\alpha$ expression or phenotype (27), and therefore both MCK-*cre*-minus $E1^f$ and $E1^{+/+}$ littermates were used as controls. $G_s\alpha$ genotyping to distinguish wild type ($E1^+$) and $E1^f$ alleles was performed by PCR using primers flanking the downstream *loxP* site (27). The presence or absence of the MCK-*cre* transgene was determined by PCR using *cre*-specific primers (27). Animals were maintained on a 12-h light/12-h dark cycle (6:00 a.m./6:00 p.m.) and standard pellet diet. Mice age 3–5 months of both sexes were used for experiments unless noted in the figure legend. The protocols were approved by the Institutional Animal Care and Use Committees 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.

Overexpression of cTnI-ND in $G_s\alpha$ -DF Cardiac Muscle—We have previously developed transgenic mouse lines overexpressing cTnI-ND in the cardiac muscle under the control of α -MHC promoter (25). Double transgenic mice were generated by mating cTnI-ND transgenic mice with MG $_s$ KO mice for cardiac functional studies. The genotype screening was done by PCR on genomic DNA isolated from tail biopsies, and all hearts used for functional studies were confirmed by Western blot for the knockdown of $G_s\alpha$ (27) and the overexpression of cTnI-ND (25).

SDS-PAGE and Immunoblot Analysis— $G_s\alpha$ protein expression in the cardiac muscle was measured on total tissue protein extracts by SDS-PAGE and Western immunoblotting using a $G_s\alpha$ -specific antibody (27). Cardiac TnI and cTnI-ND were examined by Western blotting using a monoclonal antibody TnI-1 (28) and a rabbit antiserum 7428 raised against the N-terminal peptide of cTnI.

Pro-Q Diamond Phosphoprotein Staining—To detect phosphorylated protein bands in SDS-gel, the manufacturer's protocol (Invitrogen) was applied with a few modifications. Briefly, SDS-polyacrylamide gels were prefixed in 50% methanol, 10% acetic acid for 45 min and transferred to fresh fixer overnight. After washing 3 times in deionized water for 10 min each, the gels were stained for 90 min in a dark container with Pro-Q Diamond stain freshly equilibrated to room temperature by vigorously shaking. After destaining three times in 20% acetonitrile, 50 mM sodium acetate, pH 4.0, for 30 min each, the gels were washed twice for 5 min each in deionized water and then scanned on a Typhoon 9410 fluorescence imager (GE Healthcare) using fluorescence mode (600 V, high sensitivity, green laser 532 nm for excitation and 560 nm long pass for emission).

Identification and Quantification of MHC Isoforms—Cardiac myosin heavy chain isoforms were separated by glycerol-SDS-PAGE and quantified as previously described (29). Total protein was extracted from cardiac muscle by direct homogenization in SDS-PAGE sample buffer. Myosin heavy chain isoforms were resolved using 8% polyacrylamide gel with a 50:1 ratio of acrylamide/bisacrylamide containing 30% glycerol prepared in 200 mM Tris-HCl, 100 mM glycine (pH 8.8), and 0.4% SDS. The upper running buffer was composed of 100 mM Tris base, 150 mM glycine, and 0.1% SDS containing 10 mM β -ME. The lower running buffer was 50 mM Tris base, 75 mM glycine, and 0.05% SDS. After running at 100 V in an icebox for 24 h, resolved protein bands were visualized by staining with Coomassie Blue R250.

Isolated Working Mouse Heart Preparation and Functional Measurements—Cardiac function of transgenic and wild type mice was measured in isolated working heart preparations (25, 30, 31). Thirty minutes after intraperitoneal injection of 100 units of heparin, the mice were anesthetized with pentobarbital sodium (100 mg/kg body weight, intraperitoneally), and the heart was rapidly isolated.

A modified 18-gauge needle 6 mm long with a thinned wall to reduce the outside diameter was used as the aortic cannula. A pin with a pointed end was placed inside of the needle as a guide to facilitate the cannulation. After establishing retrograde perfusion, a modified 16-gauge needle was used to cannulate the pulmonary vein for antegrade perfusion through the left atrium. A beveled PE-50 tubing was used to cannulate the pulmonary artery to collect coronary flow.

After all cannulations were established, the heart was switched to working mode by opening the left atrial perfusion. In all experiments, the hearts were perfused with Krebs-Henseleit buffer aerated with 95% O $_2$, 5% CO $_2$ at 37 °C without recycling to exclude the effects of metabolic chemicals and hormones. The buffer contents were modified as follows: 118 mM NaCl, 4.7 mM KCl, 2.25 mM CaCl $_2$, 2.25 mM MgSO $_4$, 1.2 mM KH $_2$ PO $_4$, 0.32 mM EGTA, 25 mM NaHCO $_3$, 15 mM D-glucose, and 2 mM sodium pyruvate, pH 7.4, adjusted at 37 °C. The high concentration of D-glucose (32) and sodium pyruvate effectively prevents contractile cycling (cyclic fluctuations) (33) caused by insufficient metabolic substrates for *ex vivo* working mouse heart (34), allowing the hearts to be functionally stable in working mode for over 2 h.

N-terminal Truncation of Cardiac TnI in β -Adrenergic Regulation

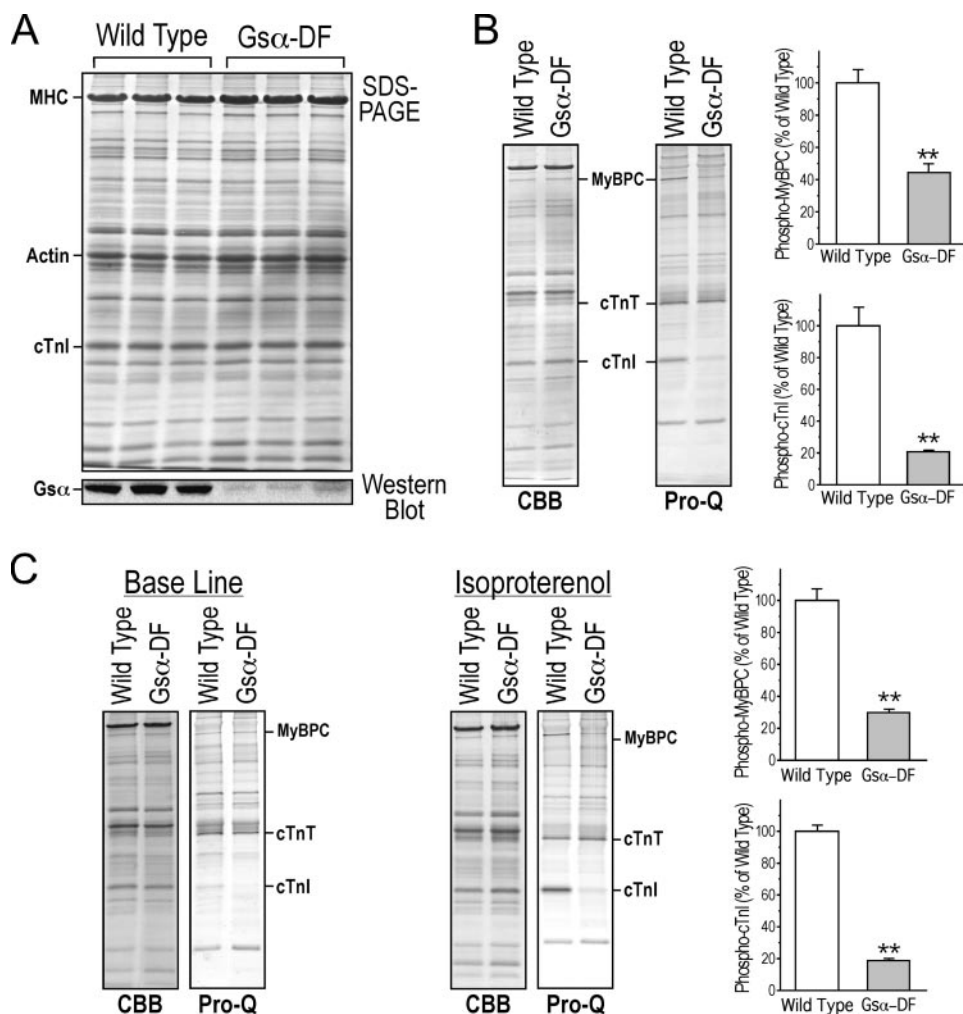


FIGURE 1. Diminished $G_s\alpha$ -protein expression and β -adrenergic signaling in $G_s\alpha$ -DF mouse hearts. A, SDS-PAGE and immunoblots of total protein extracted from wild type and $G_s\alpha$ -DF hearts using a $G_s\alpha$ -specific antibody showed $G_s\alpha$ deficiency in $G_s\alpha$ -DF hearts. B, Coomassie Brilliant Blue (CBB) and Pro-Q Diamond phosphoprotein staining of SDS-polyacrylamide gels of total protein extracts from freshly isolated hearts and densitometric quantification showed that the *in vivo* phosphorylation of cTnI and myosin-binding protein C (MyBPC) is significantly lower in the $G_s\alpha$ -DF ($n = 3$) than that of wild type ($n = 4$) mouse hearts (**, $p < 0.01$). In contrast, the non-PKA-based phosphorylation of cardiac troponin T (cTnT) had no apparent change. C, although *ex vivo* perfusion of isolated working hearts significantly reduced the phosphorylation of cTnI and MyBPC, 10 nM isoproterenol treatment increased the phosphorylation of cTnI and MyBPC in wild type ($n = 5$) but not $G_s\alpha$ -DF ($n = 4$) hearts (**, $p < 0.01$). Since the SDS-gel samples were prepared by homogenization of the cardiac muscle, the isoproterenol-treated and control samples were from different working hearts.

A 30-gauge needle was used to puncture the left ventricle wall from the apex to make a path for the insertion of a 1.2 French pressure-volume (P-V) catheter (model 898B; Scisense, London, Ontario, Canada; calibrated for pressure and volume at 37 °C). Aortic pressure was measured using an MLT844 pressure transducer (Capto, Horten, Norway). An air bubble was placed in the compliance chamber to mimic *in vivo* arterial compliance. The size of the bubble affects the aortic pressure trace and the shape of the left ventricular P-V loop. Based on conditions established by previous studies, we kept the bubble size at 0.5 ml in all experiments for isolated working mouse heart, which resembled P-V loops similar to that recorded *in vivo* (35).

Heart rate was controlled at 480 or 420 beats/min by supraventricular pacing using an isolated stimulator (A365; World Precision Instruments) with two microplatinum electrodes touching on the right atrium. Cardiac outputs were

measured by the actual aortic and pulmonary artery flows recorded in real time by calibrated drop counting using a pair of copper electrodes (one was attached to an iron clip to produce a different potential from the other) feeding to computer software (Chart 5; AD Instruments) via a Powerlab/16 SP digital data archiving system (AD Instruments). Base-line function of the isolated working hearts was measured for the intraventricular pressure, the maximum rate of left ventricular pressure development ($\pm dP/dt$ max), and left ventricular volume measurements. Stroke volume (μ l/mg heart tissue) was calculated from the sum of aortic flow and coronary effluent, normalized to heart rate.

Cardiac Function at Various Pressure Loads—During stabilization of the mouse working heart preparations and the measurement of base-line functions, the preload pressure was 10 mm Hg, and the afterload was at 55 mm Hg (25, 30, 31). To evaluate the effect of $G_s\alpha$ -DF on cardiac function against pressure load ventricular performance was measured while increasing the afterload from 55 to 90 mm Hg.

Data Analysis—Densitometry analysis of SDS-gel and Western immunoblot was performed on images scanned at 600 dots/inch using NIH Image 1.61 software. All cardiac functional analyses were performed in a blinded setting. Quantitative data were documented as mean \pm S.E. The statistical significance of differences between the mean values was analyzed by two-tail unpaired Student's *t* test unless noted in the table or figure legends.

RESULTS

$G_s\alpha$ Deficiency in Cardiac Muscle Results in Heart Failure Phenotypes with Diminished β -Adrenergic Response—Immunoblots of ventricular muscle homogenates demonstrated that the level of $G_s\alpha$ protein was significantly diminished in the MCK-cre, $E1^{fl/fl}$ (MG_sKO) mouse hearts as compared with that in wild type controls (Fig. 1A). The weak residual level of $G_s\alpha$ detected by immunoblot in the total heart tissue samples may be from noncardiac muscle tissues, such as the vasculature, nerves, and fibroblasts.

Cardiac TnI and myosin-binding protein C are two major substrates of PKA downstream of the $G_s\alpha$ signaling pathway.

TABLE 1

Base-line function of *ex vivo* working hearts

Functional studies were performed *ex vivo* in working heart preparations at 10 mm Hg preload and 55 mm Hg afterload. Heart rates were controlled by supraventricular pacing. The $G_s\alpha$ -deficient hearts showed hypertrophy with no change in left ventricle end diastolic volume. The functional parameters of $G_s\alpha$ -deficient hearts demonstrate decreased myocardial contractility. LVP_{max}, left ventricular maximum pressure; LVP_{min}, left ventricular minimum pressure; LVEDV, left ventricular end diastolic volume; TP₅₀, time to reach 50% peak pressure; RT₇₅, time to 75% relaxation.

	Wild type (n = 8)	Gs α -DF (n = 7)
Body weight (BW) (g)	29.97 ± 1.70	26.27 ± 1.29
Heart weight (HW) (mg)	141.59 ± 5.41	152.49 ± 6.78
HW/BW (mg/g)	4.77 ± 0.16	5.81 ± 0.08 ^a
+dP/dt (mm Hg/s)	3745.88 ± 210.56	2134.57 ± 141.48 ^a
-dP/dt (mm Hg/s)	2912.63 ± 106.75	2046.71 ± 101.12 ^a
LVP _{max} (mm Hg)	70.96 ± 1.67	62.66 ± 1.84 ^a
LVP _{min} (mm Hg)	0.92 ± 0.48	0.97 ± 0.81
LVEDV (μ l)	35.89 ± 1.58	36.40 ± 3.01
Stroke volume (μ l/mg)	0.111 ± 0.007	0.043 ± 0.001 ^a
Ejection fraction (%)	44.65 ± 4.19	18.81 ± 2.35 ^a
TP ₅₀ (ms)	18.94 ± 0.57	22.86 ± 0.68 ^a
RT ₇₅ (ms)	47.75 ± 1.11	48.57 ± 5.54

^a p < 0.01 versus the wild type control.

Phosphoprotein staining showed decreased phosphorylation level of the two proteins *in vivo* in $G_s\alpha$ -DF hearts, whereas the non-PKA-based phosphorylation of cardiac troponin T had no apparent change (Fig. 1B). Although *ex vivo* perfusion of isolated working hearts significantly reduced the phosphorylation of cTnI and myosin-binding protein C, reflecting the removal of sympathetic nervous tone, isoproterenol treatment increased their phosphorylation levels in wild type hearts but had no effect on that in $G_s\alpha$ -DF hearts (Fig. 1C). Altogether, the diminished PKA phosphorylation confirmed that myocardial β -adrenergic signaling was impaired in this model.

Function of the $G_s\alpha$ -DF hearts was examined *ex vivo* in isolated working heart preparations. The heart rate was controlled in the physiological range from 420 to 480 beats/min by supraventricular pacing. Summarized in Table 1, the $G_s\alpha$ -DF hearts showed hypertrophy (increased heart/body weight ratio) without significant dilation, since there was no change in the left ventricular end diastolic volume. Cardiac function analysis at 10 mm Hg preload and 55 mm Hg afterload demonstrated, among multiple parameters measured, decreased contractile and relaxation velocities ($\pm dP/dt$), left ventricle maximum pressure, time to 50% peak pressure, mean aortic pressure (diastolic pressure + 1/3 pulse pressure; Fig. 2A), stroke volume, and ejection fraction, clearly indicating decreased cardiac function.

The failing phenotype of $G_s\alpha$ -DF hearts was further demonstrated by the change of pressure-volume loop when increasing the afterload from 55 to 90 mm Hg. After raising the afterload hydrostatic column, wild type mouse hearts were able to reach 90 mm Hg afterload and produce aortic output. In contrast, not all $G_s\alpha$ -DF hearts were able to reach 90 mm Hg afterload. Functional analysis of eight wild type and five $G_s\alpha$ -DF hearts that were able to overcome 90 mm Hg afterload further showed that $G_s\alpha$ deficiency resulted in more clear decrease in cardiac function at the increased afterload (the parameters measured are shown in Table 2), consistent with a decreased myocardial potential. Representative P-V loops during the increase in afterload are shown in Fig. 2B. End systolic pressure volume relationship for the slope of changing end systolic pressure volume values quantitatively demonstrated the significantly lower

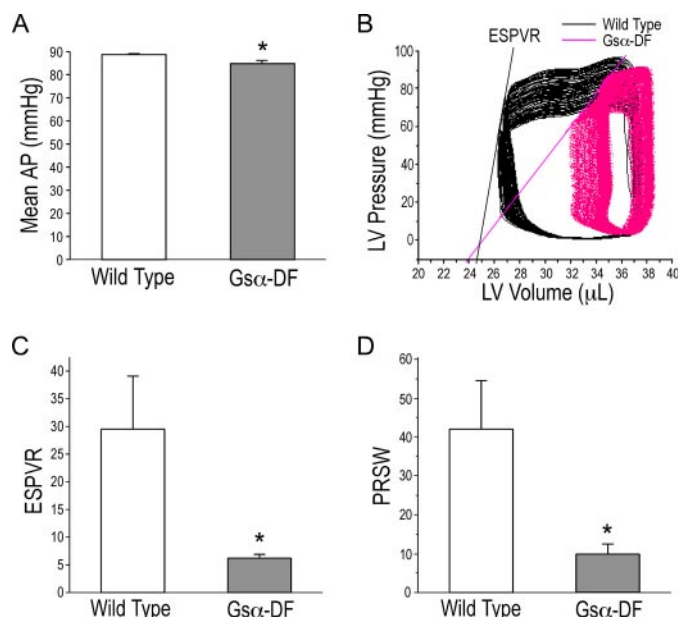


FIGURE 2. Inability of $G_s\alpha$ -DF hearts to overcome pressure overload. A, mean aortic pressure (AP) at an afterload of 90 mm Hg was lower in $G_s\alpha$ -DF than that in wild type hearts. B, P-V loops of wild type and $G_s\alpha$ -DF *ex vivo* working hearts during the increasing of afterloads from 55 to 90 mm Hg. Lines indicate the end systolic pressure volume relationship (ESPVR), and their slopes are shown in C. D, pressure recruitment of stroke work (PRSW) in wild type and $G_s\alpha$ -DF hearts was derived from P-V loop measurements during the increasing of afterloads from 55 to 90 mm Hg and showed decreased stroke work in $G_s\alpha$ -DF hearts. *, p < 0.05 versus wild type; n = 8 for wild type and n = 7 for $G_s\alpha$ -DF groups. LV, left ventricular.

TABLE 2

Isolated working heart function at increased afterload

In comparison with the base-line function measured at 55 mm Hg afterload (Table 1), cardiac function in response to pressure load was examined by increasing the afterload to 90 mm Hg. Although all wild type hearts were able to reach 90 mm Hg afterload to produce aortic output, only five of seven $G_s\alpha$ -DF hearts tested were able to overcome 90 mm Hg afterload with significantly decreased function. LVP_{max}, left ventricular maximum pressure; LVP_{min}, left ventricular minimum pressure; LVEDV, left ventricular end diastolic volume.

	Wild type (n = 8)	Gs α -DF (n = 5)
+dP/dt (mm Hg/s)	4605 ± 257	3029 ± 280 ^a
-dP/dt (mm Hg/s)	4294 ± 160	3016 ± 173 ^a
LVP _{max} (mm Hg)	99.13 ± 2.08	89.42 ± 2.45 ^b
LVP _{min} (mm Hg)	0.46 ± 0.53	0.77 ± 1.08
LVEDV (μ l)	35.86 ± 1.62	40.05 ± 4.31
Stroke volume (μ l/mg)	0.094 ± 0.008	0.032 ± 0.002 ^a
Ejection fraction (%)	37.95 ± 4.02	12.89 ± 1.05 ^a

^a p < 0.01 versus the wild type control.

^b p < 0.05 versus the wild type control.

myocardial contractility of $G_s\alpha$ -DF hearts (Fig. 2C). Pressure recruitment of stroke work derived from P-V loop measurement also showed decreased stroke work in $G_s\alpha$ -DF hearts during the increase in afterload (Fig. 2D).

Isoproterenol treatment of the working heart further confirmed impaired β -adrenergic signaling in the $G_s\alpha$ -DF hearts (Fig. 3). The intrinsic heart rate of $G_s\alpha$ -DF hearts was responsive to the perfusion of 10 nM isoproterenol, similar to that of wild type hearts, indicating unaffected β -adrenergic signaling in the pacemaker cells (Fig. 3A). Isoproterenol produced significantly positive inotropic and lusitropic effects in wild type working hearts with increased contractile velocity (+dP/dt), relaxation velocity (-dP/dt), left ventricular maximal pressure, stroke volume, and ejection fraction and reduced left ventricu-

N-terminal Truncation of Cardiac TnI in β -Adrenergic Regulation

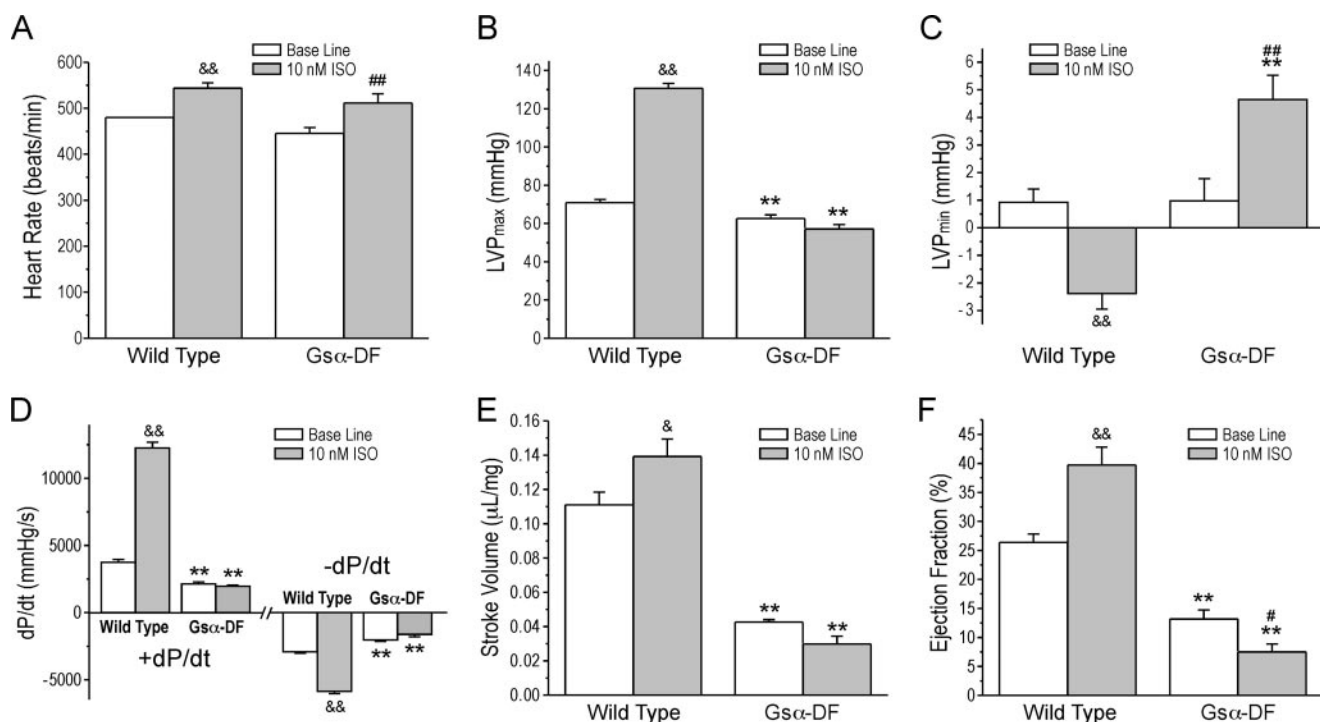


FIGURE 3. Impaired β -adrenergic responsiveness in G α -DF hearts. The data summarize intrinsic heart rate (A), maximal left ventricular pressure (LVP) (B), minimal left ventricular pressure (C), contractile (+dP/dt) and relaxation (−dP/dt) velocities (D), stroke volume (E), and ejection fraction (F) in wild type and G α -DF hearts in the absence (Base Line) or presence of 10 nM isoproterenol (ISO). *, $p < 0.05$; **, $p < 0.01$ versus the wild type control; &, $p < 0.05$; &&, $p < 0.01$ isoproterenol versus base line in wild type hearts; #, $p < 0.05$; ##, $p < 0.01$ isoproterenol versus base line in G α -DF hearts; $n = 8$ for wild type and $n = 7$ for G α -DF groups.

lar minimal pressure. In contrast, isoproterenol had no effects or opposite effects on those functional parameters in the G α -DF hearts (Fig. 3, B–F).

G α -DF Mouse Hearts Show an Increased Production of cTnI-ND—An interesting change in the myofilament proteins of G α -DF hearts was an increase of a cTnI fragment (Fig. 4A). Using monoclonal antibody TnI-1 against the C terminus of TnI and an anti-cardiac TnI N-terminal peptide rabbit polyclonal antibody 7428, immunoblots showed that this cTnI fragment lacked the N-terminal segment, whereas it retained an intact C terminus (Fig. 4B), indicating that the cTnI fragment was produced by restricted N-terminal truncation, similar to the cTnI fragment produced in rat hearts during hemodynamic adaptation to simulated microgravity (24). Quantification of the immunoblots showed that impaired β -adrenergic signaling increased the level of cTnI from 11.6 to 27.5% of total cTnI (Fig. 4C). This restricted proteolysis selectively removes the cTnI-specific N-terminal extension, including the PKA phosphorylation sites Ser²³ and Ser²⁴, retaining the functional core structure (24).

Overexpression of cTnI-ND Positively Affects the Function of G α -DF Failing Hearts—We have previously created transgenic mice overexpressing cTnI-ND in the heart to demonstrate effects on increasing cardiac muscle relaxation and improving ventricular filling (25). To investigate the functional significance of the increased cTnI-ND in G α -DF failing hearts, we generated double transgenic mice that overexpress cTnI-ND in the G α -DF hearts (Fig. 5A). Segregation of the cTnI-ND, G α -floxed, and MCK-cre alleles showed independent Mendelian patterns, indicating their locations on different chromosomes.

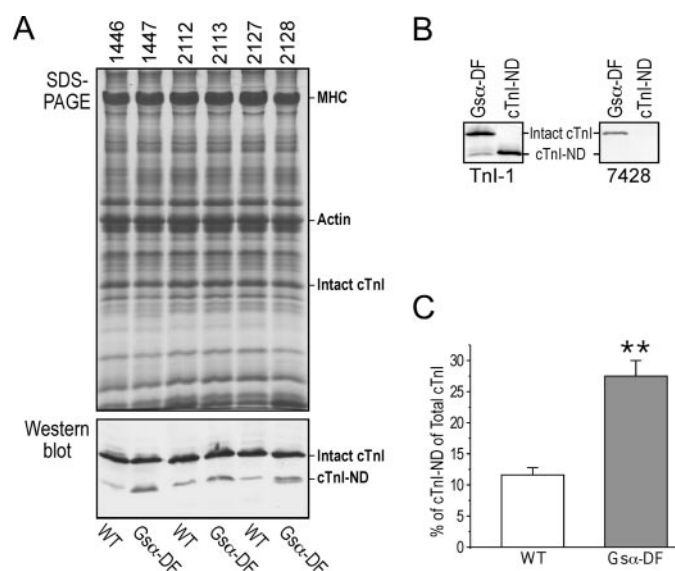


FIGURE 4. Increased levels of cTnI-ND in G α -DF hearts. A, ventricular muscle of wild type (WT) and G α -DF mice was examined by SDS-PAGE (top) and immunoblot using monoclonal antibody TnI-1 against the C terminus of TnI (bottom). The positions of intact cTnI and cTnI-ND are indicated. B, immunoblots using monoclonal antibody TnI-1 or polyclonal antibody 7428 against the N-terminal peptide of cTnI on G α -DF heart tissue and engineered cTnI-ND control protein expressed in *E. coli*. The nonreactivity of the up-regulated cTnI fragment in G α -DF hearts to antibody 7428 indicates an N-terminal truncation. C, densitometry quantification of cTnI-ND versus total cTnI in wild type and G α -DF hearts (**, $p < 0.01$; $n = 3$ in each group).

Immunoblots confirmed the diminished G α protein and overexpression of cTnI-ND in the cardiac muscle of the double transgenic mice (Fig. 5B).

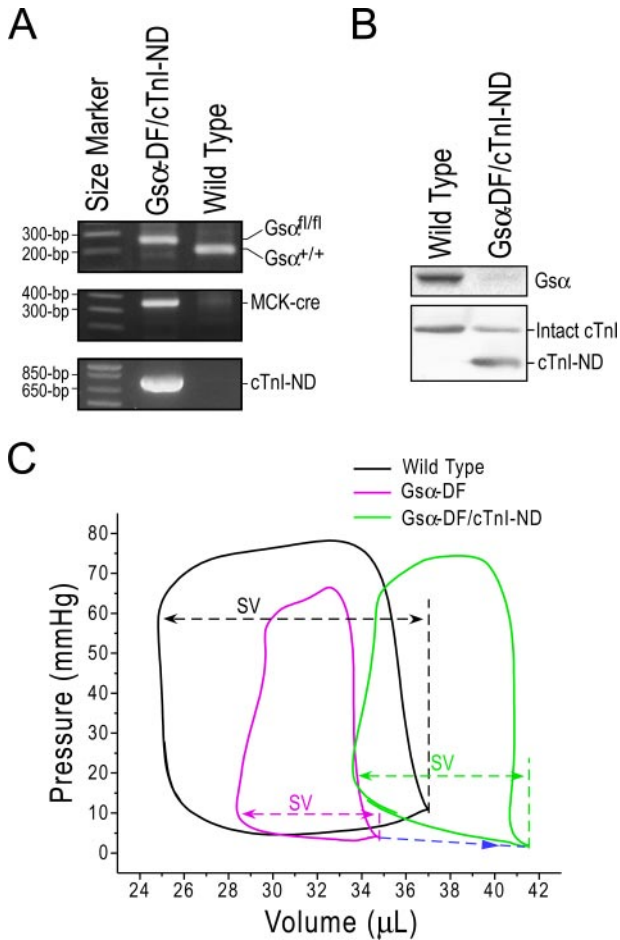


FIGURE 5. Overexpression of cTnl-ND in $G_s\alpha$ -DF mouse hearts and functional effects. A, PCR genotyping using $G_s\alpha$ -specific (top), *cre*-specific (middle), and cTnl-ND-specific (bottom) primers demonstrated the generation of double transgenic mice to overexpress cTnl-ND in $G_s\alpha$ -DF cardiac muscle. B, immunoblots with $G_s\alpha$ -specific (top) and cTnl-specific (bottom) antibodies confirmed the loss of $G_s\alpha$ and overexpression of cTnl-ND in $G_s\alpha$ -DF/cTnl-ND hearts. C, representative left ventricular P-V loops showed reduced stroke volume (SV) in $G_s\alpha$ -DF hearts in comparison with the wild type controls. Overexpression of cTnl-ND in the $G_s\alpha$ -DF hearts partially resumed the stroke volume together with increased end diastolic volume (indicated by the blue arrow).

Functional characterization using isolated working heart preparations showed that overexpression of cTnl-ND in $G_s\alpha$ -DF mouse hearts did not significantly alter ventricular contractile velocity (+dP/dt) and minimal left ventricle pressure but increased ventricular relaxation velocity (−dP/dt), maximum left ventricle pressure, maximum aortic pressure, left ventricular end diastolic volume, and stroke volume, all consistent with the established role of cTnl-ND in better utilization of the Frank-Starling mechanism (25) (Table 3). The overexpression of cTnl-ND produced a trend of myocardial hypertrophy (Table 3), suggesting a consequence of enhanced contractility. The representative P-V loops shown in Fig. 5C demonstrate the decreased maximum left ventricle pressure and stroke volume, reflecting heart failure in the $G_s\alpha$ -DF hearts and the partial reversal of these parameters as well as left ventricular end diastolic volume by overexpression of cTnl-ND. These results show that cTnl-ND overexpression partially corrected the heart failure in $G_s\alpha$ -DF mice and support the hypothesis that the increase in cTnl-ND expression in $G_s\alpha$ -DF hearts is a compensatory adaptation against the heart failure phenotype originating from impaired β -adrenergic signaling.

TABLE 3

Ex vivo function of $G_s\alpha$ -DF/cTnl-ND mouse hearts

Function of $G_s\alpha$ -DF/cTnl-ND hearts was compared with that of $G_s\alpha$ -DF hearts for the effect of cTnl-ND overexpression. Hearts of 3–5-month-old mice were tested in *ex vivo* working heart preparations. The $G_s\alpha$ -DF/cTnl-ND hearts showed hypertrophic adaptations with increased relaxation velocity, left ventricular end diastolic volume, left ventricular maximum pressure, and stroke volume. The result demonstrated that overexpression of cTnl-ND in $G_s\alpha$ -DF hearts partially corrected the failing phenotype of β -adrenergic deficiency. LVP_{max}, left ventricular maximum pressure; LVP_{min}, left ventricular minimum pressure; LVEDV, left ventricular end diastolic volume; AP_{max}, aortic maximum pressure.

	$G_s\alpha$ -DF (n = 4)	$G_s\alpha$ -DF/cTnl-ND (n = 5)
Heart weight/body weight (mg/g)	6.42 ± 0.19	8.44 ± 0.72 ^{a,b}
Paced heart rate/min	420	420
+dP/dt (mm Hg/s)	2374.67 ± 68.23	2496.40 ± 218.94
−dP/dt (mm Hg/s)	2166.67 ± 139.17	2692.80 ± 46.47 ^c
LVP _{max} (mm Hg)	68.76 ± 1.47	77.10 ± 0.78 ^c
LVP _{min} (mm Hg)	3.38 ± 1.68	2.71 ± 0.69
AP _{max} (mm Hg)	65.93 ± 0.90	68.68 ± 0.26 ^c
Stroke volume (μl/mg)	0.045 ± 0.004	0.071 ± 0.006 ^a
LVEDV (μl)	34.67 ± 1.19	42.53 ± 2.59 ^d

^a p < 0.05 versus $G_s\alpha$ -DF controls by two-tailed unpaired Student's *t* test.

^b n = 7 for $G_s\alpha$ -DF and n = 9 for $G_s\alpha$ -DF/cTnl-ND.

^c p < 0.01 versus $G_s\alpha$ -DF controls by two-tailed unpaired Student's *t* test.

^d p < 0.05 versus $G_s\alpha$ -DF by one-tailed unpaired Student's *t* test.

The $G_s\alpha$ -DF/cTnl-ND hearts also showed diminished or negative responses to the treatment of 10 nM isoproterenol, despite the improved base-line function in comparison with that of $G_s\alpha$ -DF hearts (Fig. 6). The results suggest that cTnl-ND compensates for cardiac function independent of β -adrenergic signaling.

Overexpression of cTnl-ND Reverses the Aberrant Expression of the β -MHC in $G_s\alpha$ -DF Hearts—Results of glycerol SDS-PAGE showed that ventricular muscle from wild type mice expresses both α - and β -isoforms of MHC until 3 days of age and thereafter only expresses the α -isoform (Fig. 7, A and B). Similar developmental transitions were seen in neonatal $G_s\alpha$ -DF and $G_s\alpha$ -DF/cTnl-ND mouse hearts. The identification of cardiac MHC isoforms in glycerol SDS-PAGE was confirmed by Western blots using monoclonal antibodies FA3 and FA2 for α -MHC and α/β -MHC, respectively (36) (Fig. 7C). Although only α -MHC was expressed in wild type adult mouse hearts, β -MHC reappeared in adult $G_s\alpha$ -DF hearts by 3 months of age (Fig. 7, A and B). This developmental pattern indicates that the expression of β -MHC was not a primary phenotype of $G_s\alpha$ deficiency but a secondary response to the development of heart failure. This hypothesis is consistent with the observation that abnormal expression of β -MHC in the hypertrophied adult ventricular muscle is thought to be a compensatory response for mechanical (37) and energetic (38) efficiency.

The expression of β -MHC in adult $G_s\alpha$ -ND hearts was diminished by the overexpression of cTnl-ND (Fig. 8A). These results are shown quantitatively in Fig. 8B. Together with the improved cardiac function, this reduced demand for β -MHC compensation supports the role of cTnl-ND in compensating for myocardial contractility under impaired β -adrenergic signaling.

DISCUSSION

$G_s\alpha$ Deficiency Results in Heart Failure—Previous studies have shown that overexpression of signaling molecules in the β_1 -adrenergic pathway causes heart failure due to the detri-

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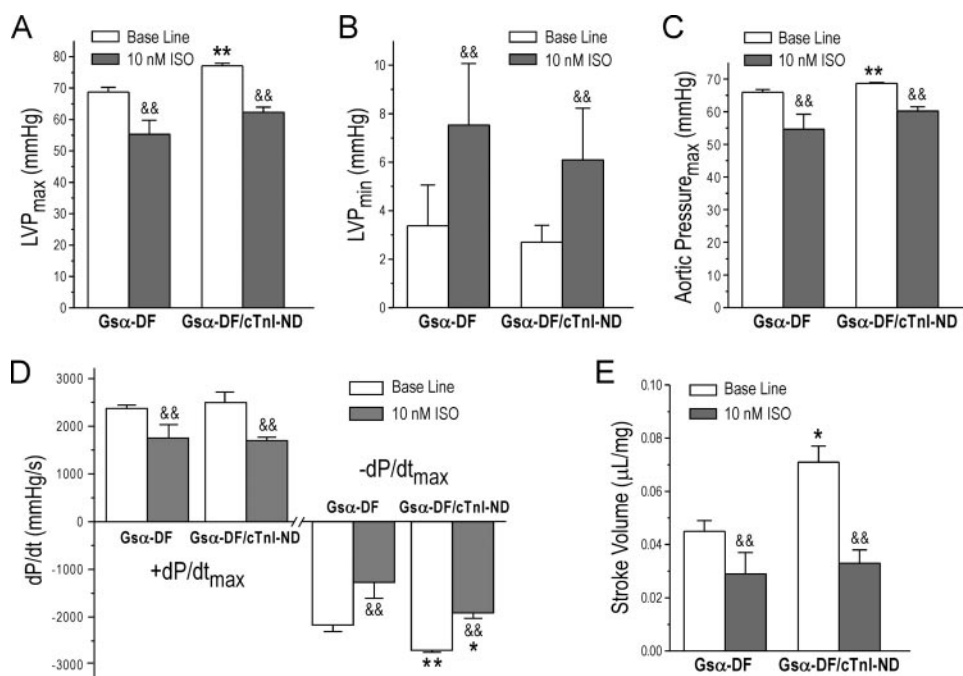


FIGURE 6. β -Adrenergic responsiveness of $G_s\alpha$ -DF/cTnI-ND double transgenic mouse hearts. The effect of 10 nM isoproterenol (ISO) on the function of $G_s\alpha$ -DF/cTnI-ND hearts was compared with that of $G_s\alpha$ -DF hearts. The functional parameters measured on *ex vivo* working hearts were as follows: maximal left ventricular pressure (LVP) (A), minimal left ventricular pressure (B), maximum aortic pressure (C), contractile (+dP/dt) and relaxation (−dP/dt) velocities (D), and stroke volume (E). The results showed that $G_s\alpha$ -DF/cTnI-ND hearts responded to 10 nM ISO treatment similar to that of $G_s\alpha$ -DF hearts. * $p < 0.05$; ** $p < 0.01$ versus $G_s\alpha$ -DF; &&, $p < 0.01$ versus base line; $n = 4$ for $G_s\alpha$ -DF and $n = 5$ for $G_s\alpha$ -DF/cTnI-ND groups.

mental effects of myocardial hypertrophy, apoptosis, and fibrosis degeneration (10–12). β_1 -AR knock-out mice survive to adulthood and appear normal but lack the chronotropic and inotropic responses to isoproterenol (39). Consistently, our present study showed that the diminished expression of $G_s\alpha$ protein in mouse cardiac muscle, which blocks β_1 -adrenergic signaling, produced depressed contractile function and heart failure phenotypes. The $G_s\alpha$ -DF failing hearts showed decreased heart functional parameters at base line and failed to overcome pressure overload.

$G_s\alpha$ -DF hearts also lacked β -adrenergic responsiveness. Phosphoprotein staining detected significantly decreased *in vivo* phosphorylation of cTnI and myosin-binding protein C (Fig. 1B). Although *ex vivo* perfusion of the working hearts significantly reduced the phosphorylation levels of cTnI and myosin-binding protein C, adding β -adrenergic agonist isoproterenol in the perfusion media very effectively restored the phosphorylation of cTnI and myosin-binding protein C in wild type hearts but had no such effect on the $G_s\alpha$ -DF hearts (Fig. 1C), indicating a lack of PKA activation. The loss of PKA activity in $G_s\alpha$ -DF cardiomyocytes is predicted to diminish the phosphorylation of proteins that regulate the Ca^{2+} handling system, including sarcolemmal L- Ca^{2+} channel, the ryanodine receptor, and phospholamban in sarcoplasmic reticulum, to negatively affect $[Ca^{2+}]_i$ of cardiac myocyte (40), which will result in decreased systolic and diastolic function in the $G_s\alpha$ -DF hearts. It has been reported that β -adrenergic stimulation activates Ca^{2+} /calmodulin-dependent protein kinase II in a non-cAMP manner to enhance cardiac muscle relaxation through phosphorylation of phospholamban (41).

Therefore, the role of this signaling pathway in $G_s\alpha$ -DF hearts remains to be investigated. Although the $G_s\alpha$ -DF mouse heart is not a perfect clinical heart failure model, it provides a valuable experimental system to investigate the *in vivo* myocardial adaptation to β -adrenergic deficiency.

Increased cTnI-ND in $G_s\alpha$ -DF Failing Hearts—cTnI-ND is produced at low levels in normal cardiac muscle and up-regulated in physiological adaptation (24). An interesting finding in the present study is that the myocardial $G_s\alpha$ deficiency-induced heart failure was accompanied by an increased level of N-terminal truncated cTnI. The PKA phosphorylation sites in cTnI are two adjacent N-terminal serine residues, Ser²³/Ser²⁴ (rat/mouse residue numbers), in the N-terminal extension. A previous study demonstrated that PKA phosphorylation increases the resistance of cardiac TnI to calpain I proteolysis (42). Consistent with the diminished

PKA activity due to $G_s\alpha$ deficiency, the level of cTnI phosphorylation was diminished in $G_s\alpha$ -DF hearts. Therefore, it is logical that diminished PKA phosphorylation in the $G_s\alpha$ -DF cardiac muscle would facilitate proteolytic modification of cTnI.

The N-terminal truncation of cTnI, which was first found in myocardial adaptation to hemodynamic changes in simulated microgravity, is a restricted proteolytic modification that preserves the core structure of TnI (24). In contrast to the proteolytic C-terminal truncation of cTnI, which removes a highly conserved segment of TnI and causes myocardial stunning (43), overexpression of cTnI-ND in transgenic mouse hearts resulted in a nondestructive cardiac phenotype with increased myocardial relaxation and improved ventricular filling for a better utilization of the Frank-Starling mechanism (25).

Proteolytic Modification of cTnI to Selectively Utilize a Downstream Mechanism of β -Adrenergic Signaling—It has been extensively demonstrated that PKA phosphorylation of the two N-terminal serines in cTnI reduces myofilament Ca^{2+} sensitivity (7, 17) and increases the rate of myocardial relaxation (18–20). The phosphorylation of cTnI is a part of the positive myocardial responses to β -adrenergic stimulation (21). Diminished PKA phosphorylation of cTnI would be predicted to decrease ventricular relaxation in the $G_s\alpha$ -DF hearts.

To test the hypothesis that cTnI-ND is a compensatory rather than a destructive response to impaired β -adrenergic signaling, we overexpressed cTnI-ND in the $G_s\alpha$ -DF hearts with a transgene and demonstrated that cTnI-ND overexpression improved cardiac function, with increased relaxation velocity, left ventricular maximum pressure, and stroke vol-

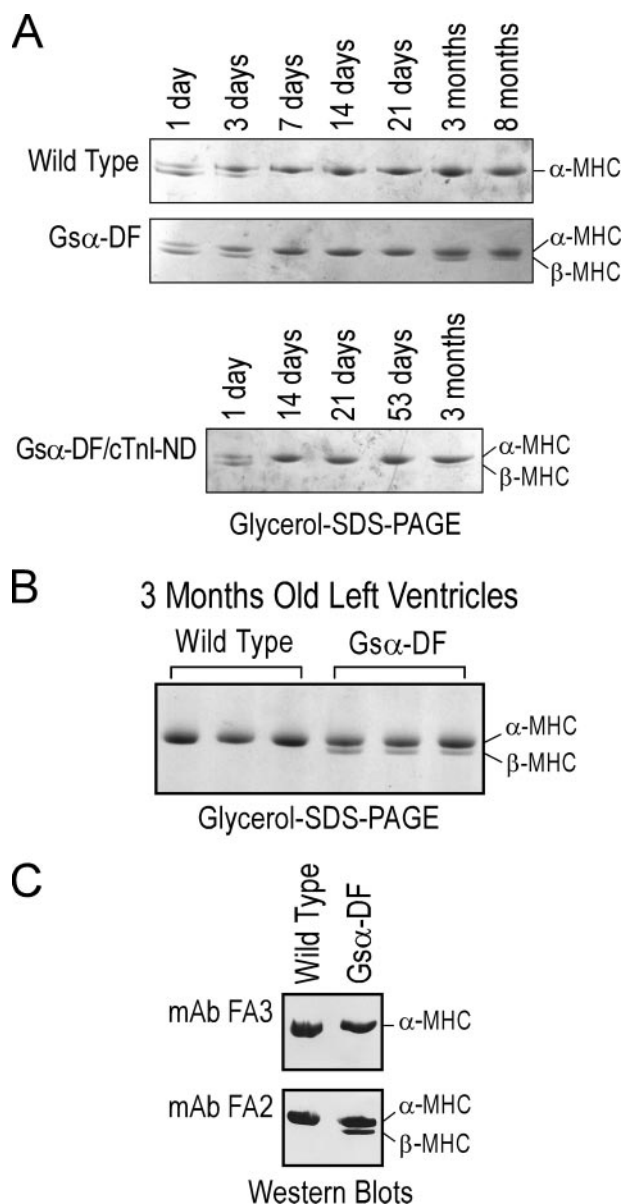


FIGURE 7. Adaptive reexpression of β -MHC in the hearts of adult $G_s\alpha$ -DF mice. *A*, the presence of α - and β -MHC isoforms was examined in wild type, $G_s\alpha$ -DF, and $G_s\alpha$ -DF/cTnI-ND mouse hearts at various ages by glycerol-SDS-PAGE. *B*, glycerol-SDS-PAGE demonstrating that only the α -MHC isoform is expressed in wild type adult mouse ventricular muscle, whereas β -MHC is re-expressed in the failing adult $G_s\alpha$ -DF hearts. *C*, immunoblotting of wild type and $G_s\alpha$ -DF hearts using monoclonal antibodies (mAb) FA3 (top) and FA2 (bottom) against α -MHC and α -/ β -MHC, respectively, confirmed the different gel mobility of α - and β -MHC isoforms in glycerol-SDS-PAGE.

ume. The results indicate that cTnI-ND could partially compensate for the decreases in myocardial relaxation velocity caused by $G_s\alpha$ deficiency, mimicking the effect of phosphorylation of intact cTnI.

The effect of proteolytic removal of the N-terminal extension of cTnI on enhancing cardiac muscle relaxation mimics that of PKA phosphorylation of Ser²³/Ser²⁴. In contrast to the broad effects of PKA phosphorylation on cardiac muscle function, the restricted N-terminal truncation of cTnI selectively utilizes a downstream mechanism of β -adrenergic signaling to enhance cardiac function without many of the side effects that precluded the use of β -agonists in the treatment of heart failure.

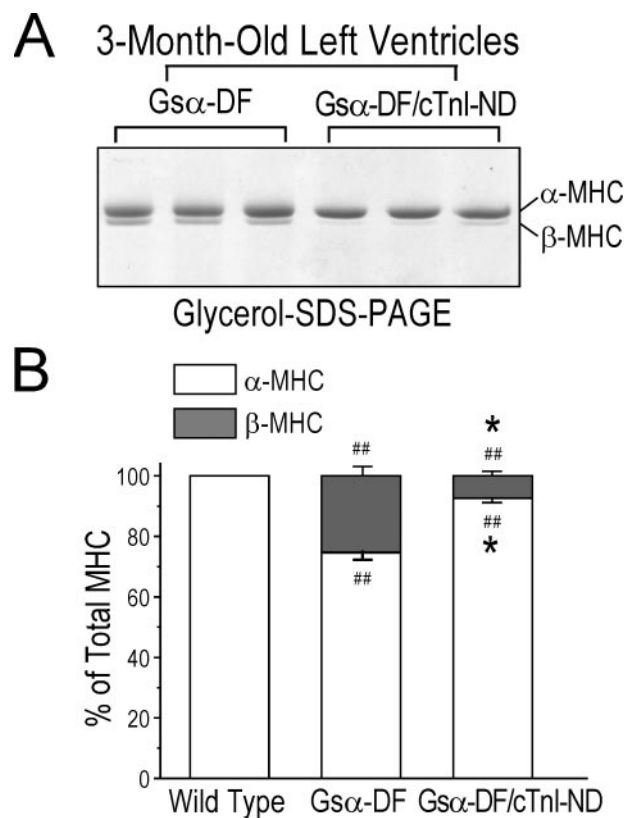


FIGURE 8. Minimized β -MHC expression in adult $G_s\alpha$ -DF mouse hearts overexpressing cTnI-ND. *A*, glycerol-SDS-PAGE demonstrating minimized expression of β -MHC in adult $G_s\alpha$ -DF/cTnI-ND double transgenic mouse hearts versus that in $G_s\alpha$ -DF hearts. *B*, densitometry analysis of immunoblots showing the percentage of α -MHC and β -MHC in adult wild type, $G_s\alpha$ -DF, and $G_s\alpha$ -DF/cTnI-ND mouse hearts confirms the compensatory effect of cTnI-ND in β -adrenergic deficient hearts (##, $p < 0.01$ versus wild type; *, $p < 0.05$ versus $G_s\alpha$ -DF; $n = 3$ in each group).

N-terminal Truncation of cTnI as a Potential Target for the Treatment of Heart Failure—Although normal adult human ventricular muscle mainly expresses β -MHC, the low level expression of α -MHC is further diminished in failing adult ventricular muscle. The proportion of α -MHC mRNA of total MHC mRNA was reduced from ~30% in nonfailing hearts to ~2% in end stage failing hearts, corresponding to a switch of 7% α -MHC protein to 100% β -MHC (44, 45). Therefore, the up-regulation of β -MHC in adult mouse hearts seen in our study serves as an indication of heart failure.

Adult ventricular muscle of small mammals expresses mainly α -MHC. When heart failure was induced in Dahl salt-sensitive rats, MHC isoforms switched from ~10% β -MHC to ~45% β -MHC (46). β -MHC is expressed in embryonic mouse heart (47), and we detected it in neonatal cardiac muscle (Fig. 7A). The expression of β -MHC in mouse heart is rapidly down-regulated after birth, and adult mice normally express 100% α -MHC in cardiac muscle. The significant reappearance of β -MHC in the 3-month-old $G_s\alpha$ -KD failing hearts, therefore, indicates an adaptive response to heart failure.

β -MHC is known to hydrolyze ATP at approximately one-half the rate of α -MHC, thus producing a lower myofibrillar ATPase activity (48). Consistently, shortening and relengthening velocities are slower in hypothyroid rat myocytes or cardiac muscle expressing increased levels of β -MHC (49, 50). A previ-

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ous study demonstrated that a shift from α -MHC to β -MHC isozymes in the mouse heart increased energy efficiency (51), suggesting a compensatory role in the case of chronic heart failure.

The level of β -MHC in $G_s\alpha$ -DF/cTnI-ND hearts was significantly lower than that in $G_s\alpha$ -DF hearts at 3 months of age. This effect of cTnI-ND overexpression further supports the functional compensation of overexpressing cTnI-ND in $G_s\alpha$ -DF failing hearts, which minimized the need for the β -MHC compensatory response. The reduced level of β -MHC in the ventricular muscle would be predicted to enhance the function, such as the maximum left ventricle pressure, of $G_s\alpha$ -DF/cTnI-ND hearts.

Since the modification of cTnI function is only a minor portion of the β -adrenergic mechanism in cardiac muscle contractility, the transgenic expression of cTnI-ND did not fully rescue the function of $G_s\alpha$ -DF hearts. It is also worth noting that the improved stroke volume of the $G_s\alpha$ -DF/cTnI-ND hearts was accompanied by a significant increase in ventricular end diastolic volume, suggesting significant left ventricular remodeling. Therefore, the long term effect on causing dilated cardiomyopathy remains to be investigated. Nonetheless, the significantly improved function of $G_s\alpha$ -DF/cTnI-ND hearts clearly demonstrated the value of increased cTnI-ND in $G_s\alpha$ -DF hearts as an endogenous adaptive response to heart failure.

In summary, the present study using genetically modified mouse models demonstrated a posttranslational modification of the thin filament regulation in cardiac muscle that compensates for the decreased cardiac function resulting from impaired β -adrenergic signaling. The restricted N-terminal truncation of cTnI forms a physiological mechanism in myocardial adaptation.

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