Enhanced N-terminal degradation of troponin I blunts cardiac function responsiveness to isoproterenol in 4-week tail-suspended rats

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Abstract. The N-terminal extension of cardiac troponin I (cTnI) is important in regulating cardiac function. Although the normal rat myocardium shows some cTnI N-terminal degradation (cTnI-ND), exposure to 4 weeks of tail-suspension markedly increased cTnI-ND. We hypothesized that the increased cTnI-ND in tail-suspended rats may affect cardiac function, particularly during β-adrenergic (β-A) stimulation. The increase in cardiac output with isoproterenol (ISO) treatment was smaller in tail-suspended rats compared with controls. Left ventricular end-diastolic pressure was elevated and increases in maximal rates of left ventricular pressure development and relaxation were lower during ISO treatment in tail-suspended rats. Response to ISO, forskolin, DB-cAMP and IBMX was also lower in cardiomyocytes from tail-suspended rats. The increase in shortening and re-lengthening the rates of cardiomyocytes at a maximal dose of ISO, forskolin, DB-cAMP and IBMX treatment was limited in tail-suspended rats. There was no difference in Ca^{2+} sensitivity of the isometric force between tail-suspended and control rats, although Ca^{2+} sensitivity was decreased less in tail-suspended rats versus control rats during PKA phosphorylation. There was no difference in PKA protein expression and activation during ISO stimulation between the two groups. Due to the increase in cTnI-ND, ISO-induced phosphorylation of cTnI was reduced in tail-suspended rats. The total phospholamban expression and phosphorylation by ISO was unaltered in tail-suspended rat hearts. These data suggest that enhanced cTnI-ND following 4-week tail-suspension is a major component of the β-A receptor signaling pathway, depressing cardiac function under ISO stimulation.

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Introduction

Troponin I (TnI) is a subunit of the troponin complex that regulates the calcium-dependent activation of myofilaments in muscle. Cardiac TnI (cTnI) exhibits a unique N-terminal extension of approximately 30 amino acids. cTnI is not present in the fast skeletal (fsTnI) or slow skeletal (ssTnI) isoforms. The N-terminal extension of cTnI contains two protein kinase A (PKA)-targeted phosphorylation sites [serine (Ser)-23 and Ser-24] (1,2). Phosphorylation of the cTnI N-terminal extension enhances contraction and accelerates relaxation during β-adrenergic (β-A) stimulation by decreased myofibril Ca^{2+} sensitivity and increased cross-bridge kinetics (3). As such, cTnI is a key regulatory protein in cardiac performance. Hearts of transgenic mice with ssTnI replacing cTnI show reduced relaxation, blunted response to β-A stimulation (4) and protection against ischemia/reperfusion injury (5), while hearts of transgenic mice with cTnI N-terminal 1-28 amino acid residue deletion show a higher baseline stroke volume and a relaxation rate, similar to wild-type mouse hearts under β-A stimulation (6). Furthermore, hearts of mice expressing cTnI-S23D/S24D (pseudo-phosphorylation at PKA sites) exhibit constitutive enhancement of rate-dependent increases in systolic and diastolic function *in vivo* (7). As well as cTnI phosphorylation, the allosteric conformation of cTnI N-terminal extension may be involved in the modulation of cardiac contractile function (8). Transgenic mice with postnatal cardiomyocyte-specific overexpression of a truncated cTnI lacking the acidic N' region (cTnI-ND2-11) exhibit significantly reduced rates of cardiac contraction and relaxation under baseline and β-agonist treatment conditions (9). Overall, these data suggest an important role of N-terminal cTnI phosphorylation in the regulation of cardiac function, particularly on the responsiveness to β-A stimulation.

The tail-suspended rat is a model used to simulate a cephalic blood shift in microgravity on the ground. The hearts of rats that have been tail-suspended for 4 weeks exhibit increased cTnI degradation, with cTnI cleaved at the 26th, 27th and 30th amino acid residues. Cardiac contractility is also decreased (10). Reduced response to isoproterenol (ISO) in the shortening amplitude and relaxation rate of cardiomyocytes has been demonstrated in 4-week tail-suspended rats (11) and is suggested to be modulated by reduced cAMP (12). However,

components of cardiac function regulation on the β-A receptor (β-AR) signal transduction pathway are unclear in the tail-suspended rat heart, particularly under β-A stimulation.

Therefore, the aim of the present study was to test our hypothesis that cTnI N-terminal degradation (cTnI-ND) in 4-week tail-suspended rats is a major component in the reduction of cardiac function responsiveness to β-A stimulation in the β-AR signal transduction pathway.

Materials and methods

Animal model. Two-month-old healthy male Sprague-Dawley rats weighing 220±10 g were used. Rats were randomly divided into control (CON) and tail-suspended (SUS) groups. There were 36 rats in each group. All the rats were housed in a 20±2˚C environment with a 12:12 h light-dark cycle and were fed rat chow and water *ad libitum*. Tail suspension was performed using the Morey-Holton method for 4 weeks (13). Care was taken to protect the tail tissue and the movement of the rats was not restricted during the procedure. All animal procedures were approved by the Animal Care and Use Committee at the Fourth Military Medical University.

Preparation of isolated working heart. Rats were injected with heparin (100 IU/100 g BW, i.p.) and anesthetized with pentobarbital sodium (40 mg/kg, i.p.). The heart was removed and the aorta was cannulated rapidly. The cannulated hearts were mounted on a heart perfusion apparatus (Radnoti Glass Technology Inc., Monrovia, CA, USA) and perfused with an oxygenated (95% O_2-5% CO_2) Krebs-Henseleit solution containing (in mM; pH 7.4) 118 NaCl, 4.7 KCl, 2.25 $MgSO_4$, 2.25 $CaCl_2$, 23.8 $NaCO_3$, 1.2 NaH_2PO_4, 0.32 EDTA and 11.5 D-glucose. Following the establishment of coronary perfusion in the Langendorff mode, the left atrium was cannulated through the pulmonary vein with a steel cannula (inner diameter, 1.8 mm; outer diameter, 2.0 mm). To detect intraventricular pressure, an ultra-miniature pressure catheter transducer (model SPR-671; Millar Instruments, Houston, TX, USA) was placed into the left ventricle through the left atrium. The preload was set at 10 mmHg. The heart was then switched from the Langendorff to the working mode at an afterload of 60 mmHg. After the working hearts were equilibrated for 30 min, they were treated with 1, 10 or 20 nM ISO (Sigma-Aldrich, St. Louis, MO, USA) for at least 5 min. Before and after ISO treatment, the aortic flow, coronary flow, heart rate, left ventricular end-systolic (LVESP) and end-diastolic (LVEDP) pressure and maximal rate of left ventricular pressure development ($+dP/dt_{max}$) and relaxation ($-dP/dt_{max}$) were measured to evaluate cardiac function of the isolated working hearts. Cardiac output is equal to aortic flow plus coronary flow. Data were obtained and analyzed using a PowerLab system and Chart software (ADInstruments Inc., Sydney, Australia).

Cardiomyocyte isolation and unloaded contractile function measurement in the single cardiomyocyte. Single ventricular myocyte isolation was performed as previously described (14). In brief, the cannulated hearts were mounted on a Langendorff perfusion apparatus and perfused with Ca^{2+}-free Joklik's modified minimum essential medium (Sigma-Aldrich) containing 10 mM HEPES and 0.1% bovine serum albumin (BSA). After 5 min, the perfusate was switched to a circulating enzyme solution containing 0.08% collagenase I (Sigma-Aldrich) for 30 min. Perfusion procedures were performed at 37˚C in a constant flow of 10 ml/min and the perfusion pressure was monitored. The ventricular tissues were chopped and the cardiomyocytes were dispersed gently by a wide-tipped pipette. The cell suspension was filtered through a 200-μm nylon mesh. Cells were resuspended in Joklik's medium containing 1% BSA after 30 min and the Ca^{2+} concentration gradually recovered to 1.25 mM.

Measurements of contractile function were performed in the Edge-Detector system (Crescent Electronics, Sandy, UT, USA) within 6 h following isolation. Cells were placed into a chamber situated on the stage of an inverted microscope (Olympus IX71; Olympus Co., Ltd., Tokyo, Japan). The cells were perfused with Tyrode's solution containing (in mM; pH 7.4) 132 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 5.0 sodium pyruvate, 10 HEPES and 10 D-glucose, at a flow rate of 0.2 ml/min at 37˚C. Electric field stimulus (15 V, 5 msec, 2.0 Hz) was administered by the stimulator. The cardiomyocytes were superfused with ISO (1, 5 and 10 nM), forskolin (0.1, 0.5 and 1.0 μM), N6,2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate sodium (DB-cAMP; 0.1, 0.5 and 1.0 mM) or 3-isobutyl-1-methylxanthine (IBMX; 50, 100 and 200 mM). Forskolin is an activator of adenylate cyclase. DB-cAMP mimics endogenous cAMP by binding to the PKA regulatory subunit and activates PKA. IBMX is a non-selective phosphodiesterase (PDE) inhibitor and cAMP is degraded by PDEs. Forskolin, DB-cAMP and IBMX were purchased from Sigma-Aldrich.

The shortening amplitude and the maximal rates of shortening ($+dL/dt_{max}$) and relaxation ($-dL/dt_{max}$) were measured by Chart software in the single cardiomyocyte.

Skinned cardiac muscle preparations. Cardiac muscle bundles (~0.2 mm in diameter and 3.6±0.4 mm in length) were dissected from the papillary muscle of the left ventricle under a dissection microscope. Bundles were mounted on a fiber apparatus (Aurora Scientific Inc., Aurora, ON, Canada) for measurement of the isometric force and the sarcomere length (High-speed Video Sarcomere Length Program, Aurora Scientific Inc.). Following equilibration for 30 min, resting force was adjusted to 100 mg, sarcomere length was not different at this initial load in any of the muscle bundles (CON, 2.01±0.04 vs. SUS, 2.03±0.07 μm). Bundles were then chemically skinned with 1% Triton X-100 for 60 min in a relaxing solution (*p*Ca 9) containing (in mM; pH 7.0) 130 potassium acetate, 1 $MgCl_2$, 5 EGTA, 5 Na_2ATP, 1X protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and 20 imidazole-HCl. A rigor solution (relaxing solution without ATP) was used to examine whether the muscle was completely skinned. Phosphorylation was performed by incubating the muscle bundles with 200 U/ml catalytic subunit of PKA (Sigma-Aldrich) in relaxing solution for 30 min. The force-*p*Ca relationship for the cardiac muscle was measured by adding $CaCl_2$ to the relaxing solution to achieve a series of *p*Ca (7.0, 6.5, 6.3, 6.0, 5.5, 5.3, 5.0 and 4.5) (15). All experiments were performed at ~22˚C.

Western blot analysis. PKA, TnI and phospholamban (PLB; total and Ser16-phosphorylated) proteins were detected by

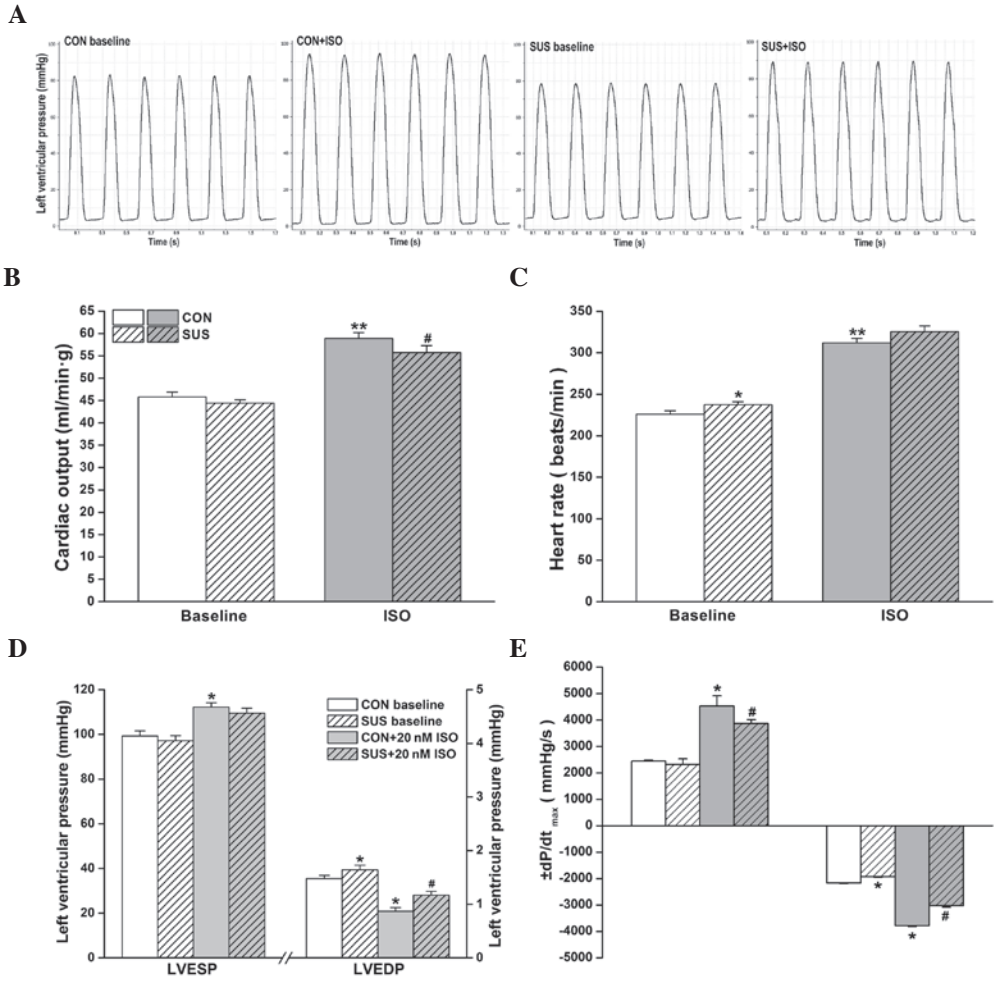

Figure 1. Contractile properties of isolated working hearts before and after ISO stimulation. (A) Representative recordings of the left ventricular pressure. (B) Cardiac output. (C) Heart rate. (D) Left ventricular end-systolic (LVESP) and end-diastolic pressure (LVEDP). (E) Maximal rates of left ventricular pressure development (+dP/dt$_{max}$) and relaxation (-dP/dt$_{max}$). Data are the mean ± SEM; n=6 hearts/group. *P<0.05 or **P<0.01 vs. baseline value of the control group. $^{\#}$P<0.05 vs. ISO-treated control group. CON, control rats; SUS, tail-suspended rats; ISO, isoproterenol.

western blot analysis. Left ventricular myocardium was homogenized in a buffer containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM DTT, 1 mM EDTA, 0.3 mM PMSF and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich). Samples were subjected to SDS-PAGE in polyacrylamide gels (12 or 14% depending on protein molecular weight). Following electrophoresis, proteins were electrically transferred to nitrocellulose membrane (0.45 μm pore size) using a Bio-Rad semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). Blotted nitrocellulose membranes were blocked with 1% BSA in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl; pH 7.5) and incubated with rabbit polyclonal anti-PKA C-α [1:10,000; Cell Signaling Technology (CST) Inc., Danvers, MA, USA], mouse monoclonal anti-TnI-I (1:4,000) (10), rabbit polyclonal anti-phosphorylated-Ser23/24 cTnI (1:1,000; CST), rabbit polyclonal anti-desmin (1:1,000; CST), mouse monoclonal anti-PLB (1:1,000; CST) and rabbit polyclonal anti-phosphorylated-Ser16 PLB (Phospho-PLB, 1:1,000; CST) in TBS containing 0.1% BSA at 4˚C overnight. Membranes were incubated in IRDye 680 CW goat anti-mouse or with IRDye 800 CW goat anti-rabbit secondary antibodies (1:10,000) for 90 min at room temperature (RT) and visualized using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA). Quantification analysis of blots was performed with the NIH Image J software (available at http://rsbweb.nih.gov/ij/download.html).

Immunofluorescent histochemistry and confocal analysis. Cardiomyocytes were incubated in a 5% CO_2 incubator at 37˚C until cells attached to the dish coated with 10 μg/ml laminin (Sigma-Aldrich). The cells were exposed to 10 nM ISO for 60 min and then fixed in 4% paraformaldehyde for 30 min. The cells were permeabilized in 0.1% Triton X-100/PBS for 30 min, blocked with 1% BSA in PBS for 60 min at RT and then incubated with rabbit polyclonal anti-PKA C-α antibody (1:100) at 4˚C overnight. The slides were rinsed twice in PBS and incubated with tetramethylrhodamine (TRITC)-labeled goat anti-rabbit IgG (1:400; Molecular Probes, Eugene, OR, USA) for 60 min. The slides were then washed in PBS, incubated in Hoechst 33258 (5 μg/ml) for 30 min and washed twice with PBS. Staining was observed using a laser-scanning confocal microscope (Olympus FV1000; Olympus Co., Ltd.) equipped with the FV10-ASW system. TRITC- and Hoechst-labeled signals were visualized at 555 and 352 nm, respectively. Images were captured at x60 water objective. Image optical densitometry analysis was performed using

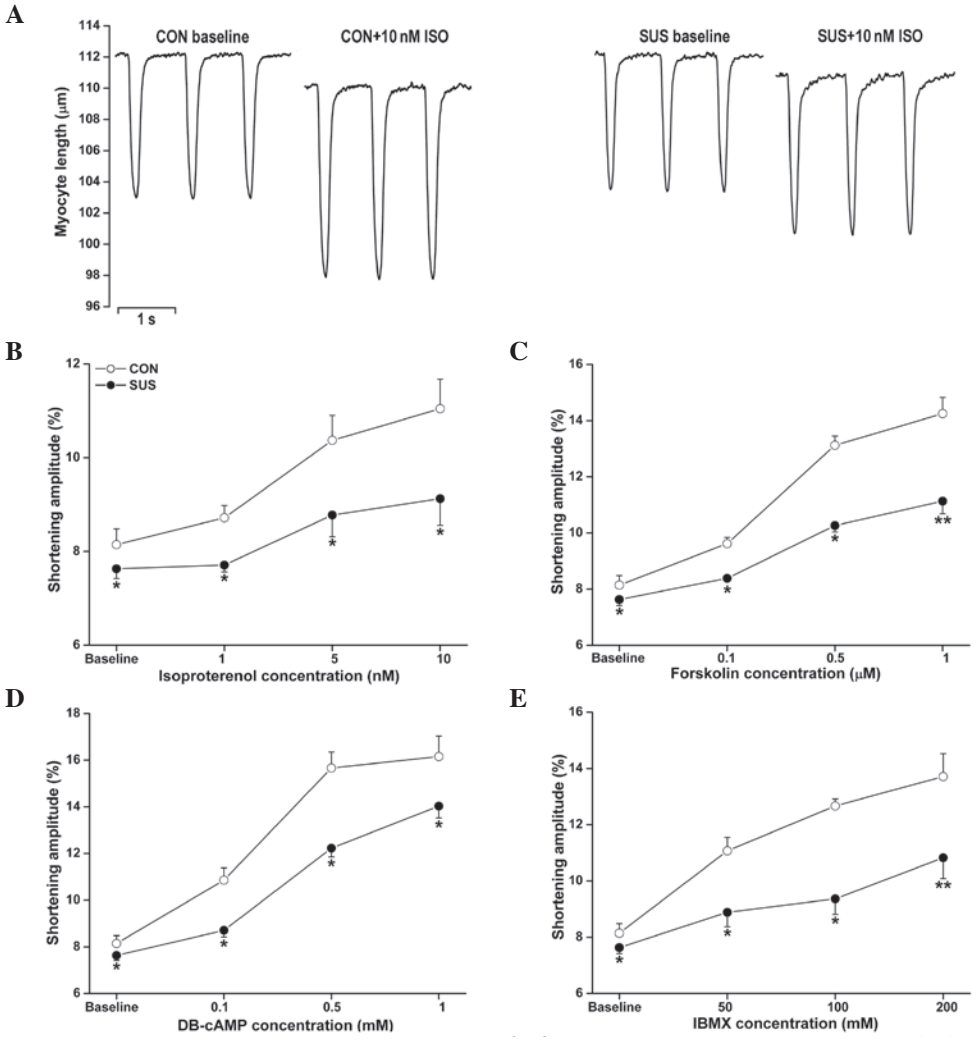

Figure 2. Unloaded contractile functions of cardiomyocytes with ISO, forskolin, DB-cAMP and IBMX treatments. Cardiomyocytes were stimulated at 2.0 Hz. The shortening amplitude of cardiomyocytes was measured in each group. (A) Representative recordings of unloaded contraction of single cardiomyocytes. (B) ISO perfusion at 1, 5 and 10 nM. (C) Forskolin perfusion at 0.1, 0.5 and 1.0 μM. (D) DB-cAMP perfusion at 0.1, 0.5 and 1.0 mM. (E) IBMX perfusion at 50, 100 and 200 mM. Data are the mean ± SEM; n=18 myocytes from six hearts for each agonist in each group. *P<0.05 or **P<0.01 vs. the control group. CON, control rats; SUS, tail-suspended rats; ISO, isoproterenol; DB-cAMP, N6,2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate sodium; IBMX, 3-isobutyl-1-methylxanthine.

Olympus Fluoview image analysis software (Olympus Co., Ltd.).

Statistical analysis. Data were presented as the mean ± SEM. Differences between the two groups were compared by the unpaired Student's t-test. For multi-group comparisons, two-way ANOVA followed by Tukey post-hoc test was performed. P<0.05 was considered to indicate a statistically significant difference.

Results

Reduced responsiveness of isolated working heart to ISO in tail-suspended rats. Body weights of tail-suspended rats were similar to those of age-matched controls (CON, 304.7±11.9 g vs. SUS, 287.5±11.9 g; P>0.05). The wet weight of the hearts in the tail-suspended rats was also not found to be significantly different from those in the control rats (CON, 930.2±37.5 mg vs. SUS, 931.9±24.3 mg; P>0.05).

Cardiac function in working heart mode was assessed at a preload of 10 mmHg and an afterload of 60 mmHg. Basal cardiac output values were similar between control and tail-suspended rats (P>0.05). ISO stimulation increased cardiac output in the control and tail-suspended groups, but there was a higher increase in cardiac output following 20 nM ISO treatment in the control compared with the tail-suspended group (Fig. 1B). The intrinsic the heart rate was 225.9±4.1 beats/min in the control group and 237.5±3.5 beats/min in the tail-suspended group. During 20 nM ISO perfusion, heart rate increased to 312±5.4 beats/min in the control and to 325.5±6.7 beats/min in the tail-suspended group. The basal heart rate was found to be significantly higher in the tail-suspended group compared with the control group (Fig. 1C).

ISO treatment induced a significant increase in LVESP of the control (P<0.05) and the tail-suspended group (P<0.05), while no difference in LVESP between the tail-suspended and control groups with or without ISO treatment was observed (Fig. 1D). Basal LVEDP was higher in the tail-suspended group than the control group (P<0.05). A significant decrease in LVEDP following perfusion with 20 nM ISO was found in the groups, which was more marked in the control than in the tail-suspended group (P<0.05, Fig. 1D).

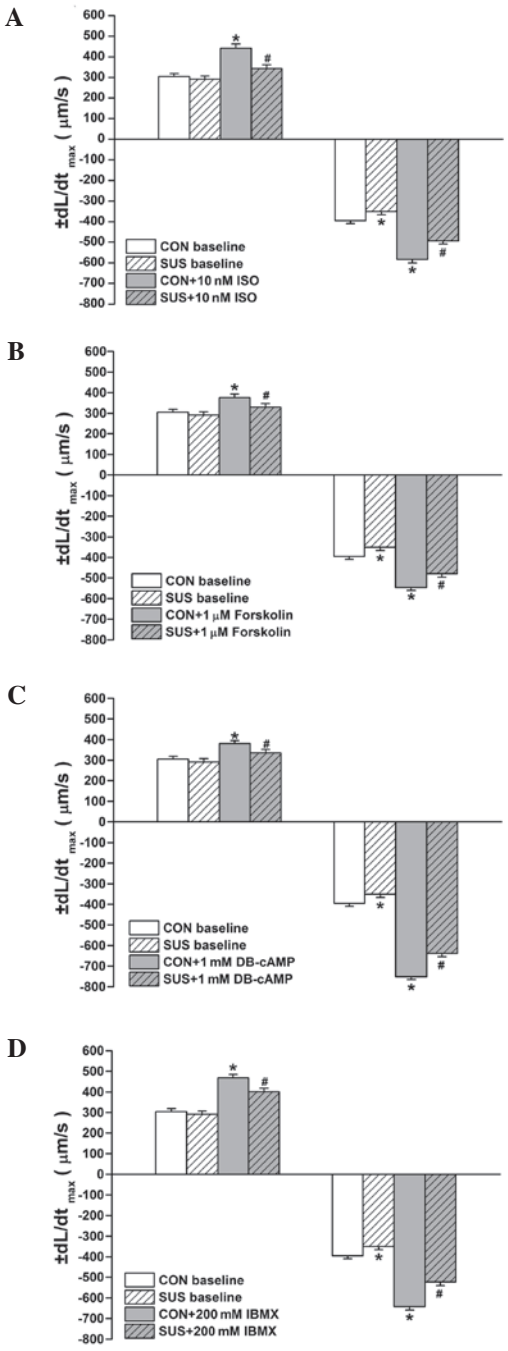

Figure 3. Effects of ISO, forskolin, DB-cAMP and IBMX on maximal rates of shortening (+dL/dt$_{max}$) and relaxation (-dL/dt$_{max}$) in cardiomyocytes. Treatment of (A) 10 nM ISO, (B) 1.0 μM forskolin, (C) 1 mM DB-cAMP, (D) 200 mM IBMX is shown. Data are the mean ± SEM; n=18 myocytes from 6 hearts for each agonist in each group. *P<0.05 vs. baseline value of the control group. #P<0.05 vs. ISO-treated control group. CON, control rats; SUS, tail-suspended rats; ISO, isoproterenol; DB-cAMP, N6,2'-*O*-dibutyryladenosine 3',5'-cyclic monophosphate sodium; IBMX, 3-isobutyl-1-methylxanthine.

Under baseline conditions, no change in +dP/dt$_{max}$ was observed in the tail-suspended group, while -dP/dt$_{max}$ was found to be significantly reduced when compared with the control group (P<0.05; Fig. 1E). Following 20 nM ISO treatment, a significant increase in +dP/dt$_{max}$ and -dP/dt$_{max}$ was found in the control (P<0.05) and tail-suspended groups (P<0.05) compared with the values prior to ISO administration. Increase in ±dP/dt$_{max}$ in response to ISO was greater in the control group than the tail-suspended group (P<0.05, Fig. 1E).

Depressed responsiveness of cardiomyocytes to β-AR signaling pathway agonists in tail-suspended rats. To determine the major mediator(s) regulating the ISO responsiveness in tail-suspended rats, we examined the effects of the upstream agonists of the β-AR signaling pathway in cardiomyocytes at a stimulation frequency of 2.0 Hz. The baseline value in shortening amplitude of cardiomyocytes was lower in the tail-suspended group than in the control group (Fig. 2). Exposure to ISO, forskolin, DB-cAMP and IBMX produced dose-dependent positive inotropic responses in control and tail-suspended rat cardiomyocytes. These responses were significantly lower in the tail-suspended group than in the control group (Fig. 2).

The increase in cardiomyocyte shortening amplitude following ISO stimulation (1, 5 and 10 nM) was less marked in tail-suspended rats compared with control rats (Fig. 2B). Forskolin (0.1, 0.5 and 1.0 μM), an activator of adenylate cyclase, induced a smaller increase in shortening amplitude in the tail-suspended group (Fig. 2C). DB-cAMP is a cell membrane-permeable and PDE-resistant cAMP analog. Exposure to 0.1, 0.5 and 1.0 mM DB-cAMP induced a smaller increase in cardiomyocyte shortening amplitude in tail-suspended rats compared with the control (Fig. 2D). IBMX (50, 100 and 200 mM), a non-selective inhibitor of PDEs, also induced a smaller increase in shortening amplitude in the tail-suspended group (Fig. 2E).

Baseline values of +dL/dt$_{max}$ of cardiomyocytes were similar between the control and tail-suspended groups, while the baseline values of -dL/dt$_{max}$ decreased significantly in the tail-suspended compared with the control group (P<0.05; Fig. 3A). ISO, forskolin, DB-cAMP and IBMX all enhanced the +dL/dt$_{max}$ and -dL/dt$_{max}$ of cardiomyocytes in the control and tail-suspended groups (P<0.05; Fig. 3). The increased +dL/dt$_{max}$ and -dL/dt$_{max}$ in response to ISO, forskolin, DB-cAMP or IBMX treatments were significantly less in the tail-suspended group than the control (P<0.05; Fig. 3).

PKA reduced myofibrillar Ca^{2+} sensitivity in the myocardium. The maximum Ca^{2+}-activated isometric force of skinned muscle fibers from control rats was significantly higher than that from tail-suspended rats (10). No difference was observed in the myocardial isometric force-*p*Ca relationship between the two groups (Fig. 4). Although PKA induced a reduction in myofibrillar Ca^{2+} sensitivity in the control and tail-suspended groups, the reduction was less marked in tail-suspended rats. The [Ca^{2+}] required for 50% activation (*p*Ca50) was 6.07±0.17 *p*Ca units at baseline and 5.93±0.09 following PKA treatment in tail-suspended rat myofibrils, compared with 6.08±0.12 *p*Ca units at baseline and 5.79±0.11 following PKA treatment in the control rats (P<0.05).

Increased N-terminal degradation of cTnI and reduced cTnI phosphorylation by ISO in the tail-suspended group. Western blot analysis revealed two bands of cTnI in hearts from the control and tail-suspended rats (Fig. 5A). The percentage of intact cTnI (upper band)/total cTnI (intact cTnI plus cTnI fragment) was decreased in tail-suspended rat hearts compared with the control (P<0.05). Optical densitometry of the cTnI

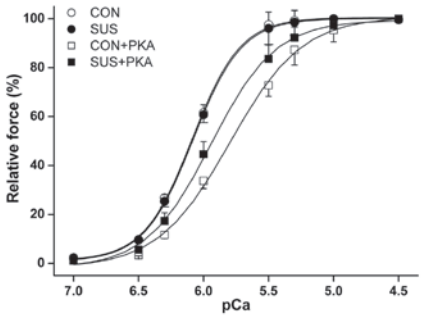

Figure 4. Effects of phosphorylation by PKA on the force-*p*Ca relationships of skinned cardiac muscles from control and tail-suspended rats. Force is expressed relative to the maximum force before PKA. Lines with the least squares fits to the Hill equation. Data are the mean ± SEM; n=6 muscle bundles from 6 hearts/group. CON, control rats; SUS, tail-suspended rats; PKA, protein kinase A.

fragment (lower band) was 15.5±1.3% of total cTnI in the control and 23.0±0.7% in the tail-suspended group (Fig. 5B), with significantly greater cTnI degradation in the tail-suspended group versus the control group (P<0.05). The basic phosphorylation level of cTnI was significantly decreased in the tail-suspended group compared with the control (P<0.05; Fig. 5A and C). Following ISO stimulation, phosphorylated cTnI increased in tail-suspended and control groups, however, the increase was smaller in the tail-suspended group than that in the control (P<0.05; Fig. 5C).

Expression and activation of PKA were unaltered in the tail-suspended group. Inactive PKA is a heterotetramer composed of a regulatory subunit (R) dimer and a catalytic subunit (C) dimer. In its inactive state, pseudosubstrate sequences on the R subunits block the active sites on the C subunits. When cAMP binds to the R subunits, the auto-inhibitory contact is relieved and active monomeric C subunits are released. Three C subunit isoforms (C-α, -β and -γ) have been identified and C-α is predominantly expressed in the heart. Thus, using PKA C-α subunit antibody we detected activated PKA. Compared with desmin, no difference in the expression of the activated PKA C-α subunit between the control and tail-suspended groups before and after 10 nM ISO stimulation was observed (Fig. 6A and B). Immunofluorescence data revealed a similar degree of PKA translocation into the sarcolemma of cardiomyocytes following 10 nM ISO stimulation between the control and tail-suspended groups (Fig. 6C and D).

Expression and Ser16 phosphorylation of PLB were unchanged in the tail-suspended group. The level of total PLB and/or phosphorylation at Ser16 (the residue phosphorylated by PKA) are important factors that may alter relaxation function in tail-suspended rat hearts. However, there was no change in the expression or phosphorylation of PLB at Ser16 by western blot analysis following ISO stimulation (Fig. 7).

Discussion

In the present study, the baseline LVEDP of the working heart was higher in the tail-suspended rats compared with the control, while a blunted response to ISO in all parameters

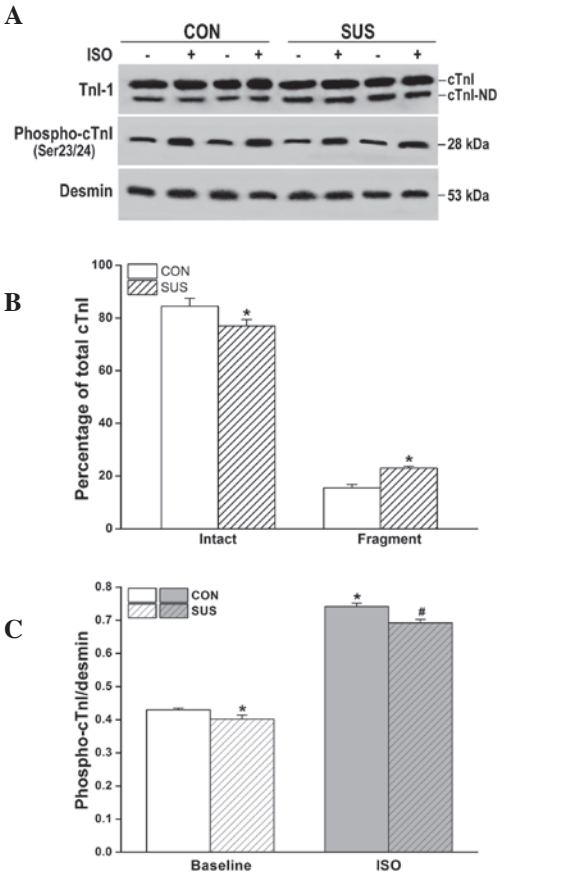

Figure 5. N-terminal degradation and phosphorylation of cTnI in control and tail-suspended rats. (A) Representative immunoblots and mean data showing (B) N-terminal degradation and (C) phosphorylation of cTnI (n=6 hearts per group). Data are the mean ± SEM. *P<0.05 vs. baseline value of the control group. #P<0.05 vs. ISO-treated control group. CON, control rats; SUS, tail-suspended rats; TnI-1, troponin I 1; cTnI-1, cardiac TnI-1.

measured in the working heart, isolated cardiomyocytes and skinned fibers was identified in tail-suspended rats. Thus, the decrease in ISO sensitivity with tail-suspension was associated with changes in the β-AR signaling pathway at the level of the individual cardiomyocyte.

Stimulation of β-AR by ISO activates G_s, which in turn activates adenylate cyclase and increases the formation of cAMP within cardiomyocytes. Elevated levels of cAMP increase activation of PKA, which phosphorylates intracellular targets, including L-type Ca^{2+} channels, ryanodine receptor (RyR), PLB, cTnI and myosin-binding protein C (MyBPC). Therefore, the upstream components in the β-AR signal transduction pathway include β-AR, G_s, adenylate cyclase, cAMP and PKA. The downstream components are the targeting proteins of PKA.

Forskolin, a direct adenylate cyclase agonist, caused a smaller increase in the shortening amplitude and the maximal rate of relaxation in tail-suspended rat cardiomyocytes. By contrast, a reduction in cAMP of cardiomyocytes has been reported to depress the response to ISO in tail-suspended rats (12). However, in the present study, response to DB-cAMP was less in the cardiomyocytes of tail-suspended rats compared with the control. In addition, under basal conditions and during β-AR stimulation, intracellular cAMP levels are regulated by PDE, which catalyzes the breakdown

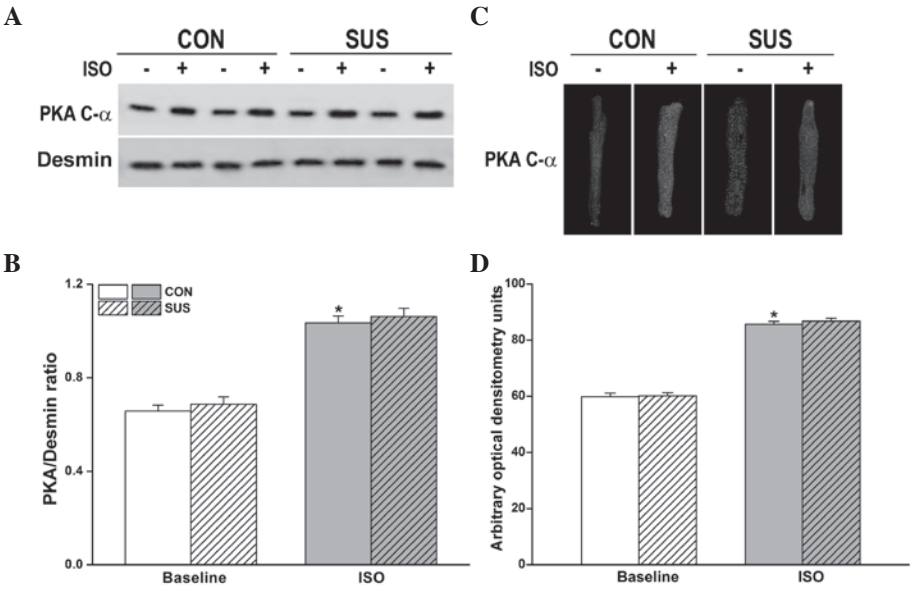

Figure 6. Expression and translocation of PKA before and after 10 nM ISO stimulation in the control and tail-suspended rats. (A and B) Representative immunoblots and mean data showing activated PKA before and after ISO stimulation. Data are the mean ± SEM; n=6 hearts per group. (C) Representative immunofluorescence of cardiomyocytes for PKA with tetramethylrhodamine, scanned at the cell membrane level. (D) Mean data showing the translocation of PKA to the membrane after ISO treatment. Data are the mean ± SEM; n=9 myocytes from 3 hearts/group. *P<0.05 vs. baseline value of the control group. CON, control rats; SUS, tail-suspended rats; ISO, isoproterenol; PKA, protein kinase A.

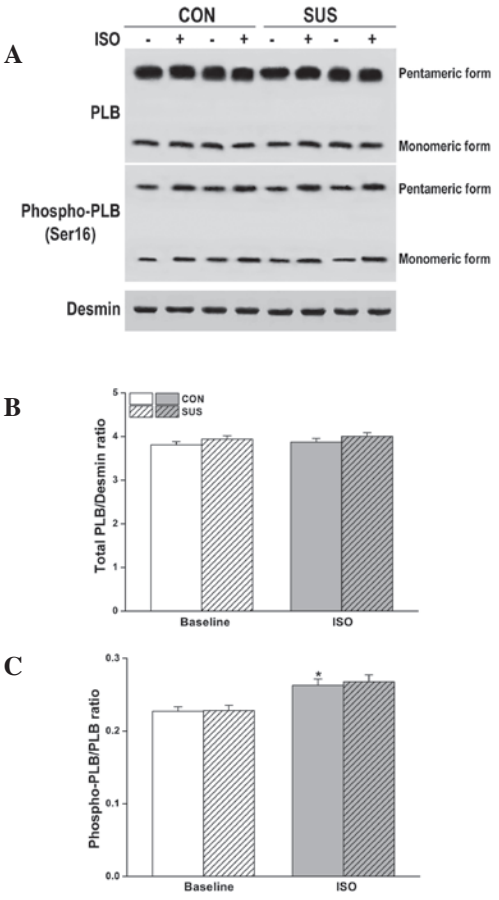

Figure 7. Expression of PLB and phospho-PLB at Ser16 before and after 10 nM ISO treatment in the control and tail-suspended rats. (A) Representative immunoblots and mean data showing the (B) expression of PLB and (C) phospho-PLB (Ser16). PLB was detected in its pentameric and monomeric forms. Data are the mean ± SEM; n=6 hearts/group. *P<0.05 vs. baseline value of the control group. CON, control rats; SUS, tail-suspended rats; ISO, isoproterenol; PLB, phospholamban; phospho-PLB, phosphorylated PLB.

of cAMP. The PDE inhibitor IBMX did not reverse the tail-suspension-related deficit in positive inotropy caused by β-AR stimulation. PKA expression and membrane translocation of cardiomyocytes during β-AR stimulation was similar in both the tail-suspended and control rats. These findings suggest that events downstream of PKA in the β-AR signal transduction pathway may be affected by the tail-suspension.

Phosphorylation of L-type Ca^{2+} channels enhances Ca^{2+} influx, increasing the shortening amplitude or force of contraction, but does not regulate the relaxation of cardiomyocytes. A previous study demonstrated that adrenergic regulation of cardiac contractility does not involve phosphorylation of the cardiac ryanodine receptor at Ser2808 by PKA (16). Phosphorylation of MyBPC does not appear to have any effect on myofibrillar Ca^{2+} sensitivity of the isometric force, which modulates the relaxation, but accelerates kinetics of force development (17). Phosphorylated MyBPC is only involved in myocardial protection during ischemia (18,19). This indicates that phosphorylation of PLB and cTnI is important in the regulation of cardiac function, particularly the relaxation function of cardiomyocytes downstream of the β-AR signal transduction pathway.

Phosphorylation of PLB ameliorates the inhibition of SERCA and increases Ca^{2+} uptake by SERCA. Thus, the rate of relaxation is enhanced during β-AR stimulation due to increased sequestration of Ca^{2+} by increased SERCA activity. In PLB knockout mice, phosphorylation of PLB has been shown to account for approximately 50% of the enhanced relaxation rate effect (20). However, there was no difference in total PLB and PLB phosphorylation at the PKA-targeted site Ser16 residue in cardiomyocytes between the tail-suspended and control rats. cTnI, another substrate phosphorylated by PKA, exhibited N-terminal degradation in cardiomyocytes of control and tail-suspended rats, although the N-terminal degradation of cTnI was more marked in tail-suspended rats. In turn,

the increased N-terminal degradation of cTnI reduced phosphorylation in tail-suspended rats. Thus, these data strongly suggest that the blunted cardiac function in β-AR stimulation is correlated with enhanced cTnI N-terminal degradation in the tail-suspended rats.

A number of mechanistic systems models have been developed to analyze the functional roles of PLB, L-type calcium channel, RyR and cTnI phosphorylation upon β-AR stimulation in rat ventricular myocytes. The model analysis revealed that the PKA-mediated phosphorylation of cTnI only exhibits a nominal lusitropic response during β-AR stimulation (21). However, transgenic mice expressing ssTnI specifically in cardiomyocytes exhibit less shortening and prolongation of the half-time of intracellular [Ca^{2+}] decay, while similar transgenic cardiomyocytes show no enhancement of the velocity of shortening during isoprenaline treatment (4). By using PLB knockout transgenic mice, cTnI phosphorylation has been shown to contribute 14-18% of the lusitropic effect during maximal isometric contractions (20). Overall, these data indicate that phosphorylation of cTnI at the N-terminus by PKA is a key modulator of cardiac function. In addition, the allosteric conformation of cTnI N-terminal extension also regulates cardiac contractile function (8). Degradation of N-terminal extension induces a change in the allosteric conformation of cTnI. Sadayappan *et al* (9) have generated transgenic mice with a truncated cTnI that lacks the acidic N' region (cTnI-ND2-11). The acidic N' region is not involved in the phosphorylated sites of cTnI, although cTnI-ND2-11 hearts exhibit significantly reduced rates of contraction and relaxation under baseline and β-agonist treatment (9).

cTnI has been previously shown to be cleaved at the 26th, 27th and 30th amino acid residues in tail-suspended rat hearts (10). N-terminal degradation of cTnI decreased the degree of total cTnI phosphorylation by PKA in the hearts of tail-suspended rats. Although cTnI-ND accounted for only approximately 20% of total cTnI in tail-suspended rats in the present study, this cTnI-ND lacked the PKA phosphorylation sites, Ser23 and Ser24, changing the allosteric conformation of cTnI N-terminal extension. These combined effects may enhance the modulation of the cardiac function in tail-suspended rat hearts. Therefore, in the present study, increased cTnI-ND reduced the contraction and relaxation function of the left ventricle in working hearts and impaired cardiac function of cardiomyocytes in tail-suspended rats at baseline and during β-AR stimulation. Barbato *et al* reported that the transgenic mouse hearts with the N-terminal truncation of cTnI reduced LVEDP at the basic level, but the response of $\pm dP/dt_{max}$ to ISO was also lower in transgenic mice than wild-type mice (6).

Furthermore, cTnI phosphorylation at Ser23 and Ser24 by PKA has been shown to depress the Ca^{2+} affinity and Ca^{2+} off-rate of cTnC *in vitro* (22). A rapid dissociation of Ca^{2+} from cTnC is another important factor that accelerates the relaxation rate during β-agonist treatment. Removal of the N-terminal domain of cTnI from 1-28 amino acid residues has been shown to decrease the Ca^{2+} sensitivity of actomyosin ATPase in the transgenic mouse myocardium (6). The lower Ca^{2+} sensitivity of the force generated by muscle fibers under β-AR stimulation is correlated with this more rapid dissociation of Ca^{2+} from cTnC. Therefore, increased dissociation of Ca^{2+} from cTnC, coupled with a more rapid uptake of Ca^{2+} by the sarcoplasmic reticulum stimulated by PKA phosphorylation of PLB, may account for the more rapid relaxation observed during the inotropic response of the heart to ISO. In the present study, we observed no difference in the Ca^{2+} sensitivity of myofibrils between the control and tail-suspended rat hearts, but the Ca^{2+} sensitivity of myofibrils exhibited less reduction following PKA treatment in tail-suspended hearts. Therefore, the working hearts and cardiomyocytes demonstrated a slow relaxation and blunted responsiveness to ISO in 4-week tail-suspended rats.

In summary, N-terminal degradation of cTnI in tail-suspended rat hearts is a major component to reduce cardiac function responsiveness to ISO in the β-AR signal transduction pathway.

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