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Nuclear Translocation of Calpain-2 Regulates Propensity Toward Apoptosis in Cardiomyocytes of Tail-Suspended Rats

Hui Chang, Lin Zhang, Peng-Tao Xu, Quan Li, Juan-Juan Sheng, Yun-Ying Wang, Yan Chen, Lan-Ning Zhang, and Zhi-Bin Yu*

Department of Aerospace Physiology, Fourth Military Medical University, 169# Changlexi Road, Xi'an 710032, China

ABSTRACT

The compensatory increase in catecholamine release does not reverse orthostatic intolerance after returning from a long-term spaceflight, but it is unclear whether high dose of catecholamine induces cardiac damage. The tail-suspended rat model was used to simulate the effects of weightlessness on the heart. Apoptotic rates in the left ventricular myocardium did not increase in 4-week of tail-suspended rats compared with the synchronous control. On the contrary, isoproterenol (intraperitoneal injection) and 1-day recovery from the 4-week tail-suspension increased apoptotic rates in the myocardium. Propranolol and PD150606 inhibited cardiomyocyte apoptosis in the recovery group. PD150606 and calpain-2 knockdown also blocked isoproterenol-induced cardiomyocyte apoptosis in tail-suspended rats. The activity and nuclear translocation of calpain-2 increased, but the expression of calpain-1, calpain-2, and calpastatin was unchanged in the myocardium of tail-suspended rats. The Ser-16-phosphorylated phospholamban of the nuclear envelope was higher in tail-suspended rats than in the control rats under isoproterenol stimulation. Isoproterenol treatment also induced a large intranuclear Ca^{2+} transient of cardiomyocytes in tail-suspended rats. These results suggest that high-dose isoproterenol phosphorylates phospholamban of the nuclear envelope and increases intranuclear Ca^{2+} transient. Larger intranuclear Ca^{2+} further activates nuclear calpain-2 and hence induces cardiomyocyte apoptosis. J. Cell. Biochem. 112: 571–580, 2011.

KEY WORDS: TAIL-SUSPENSION; CARDIOMYOCYTE; APOPTOSIS; CALPAIN-2; NUCLEAR TRANSLOCATION

The most profound changes that occur in man during prolonged exposure to weightlessness environment in space are a cephalic redistribution of fluid, a reduction in plasma volume (hypovolemia), and selective increases in beta1- and beta2-adrenoreceptor responsiveness in the heart [Convertino et al., 1997a,b]. The tail-suspended rat is a widely used animal model to simulate the effects of weightlessness. This rat model can simulate weightlessness-induced changes in the cardiovascular system [Martel et al., 1998]. The cardiovascular abnormality manifested by orthostatic intolerance on return from space is due, at least in part, to a reduction in cardiac contractility and plasma volume [Convertino et al., 1997a; Yu et al., 2001]. It is unclear whether a compensatory increase in catecholamine release during the recovery period and high beta-adrenoreceptor responsiveness in the heart induces cardiac damage. Hypovolemia induced by weightlessness reduces the pre-load of the heart. Mechanical unloading (transplant) of the heart activates the calpain system in the myocardium [Razeghi et al., 2007]. A high concentration of catecholamine is also a stress-factor which results in activation of calpain in the heart [Li et al., 2009a]. An increase in the N-terminal deletion of cardiac troponin I (cTnI) suggests that there is enhanced calpain activity in 4 weeks of tail-suspended rat hearts [Yu et al., 2001].

Ubiquitous calpains are Ca^{2+}-dependent cysteine proteases composed of two molecules: calpain-1 (μ-calpain) and calpain-2 (m-calpain). In addition, there is an endogenous inhibitor, calpastatin [Goll et al., 2003]. Myocardium contains moderate amounts of calpain-1, calpain-2, and calpastatin [Galvez et al., 2007]. The micromolar Ca^{2+}-activated calpain-1 and millimolar Ca^{2+}-activated calpain-2 are implicated in cardiomyocyte apoptosis in the diabetic and ischemia-reperfusion (IR) heart [Chen et al., 2001; Li et

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Hui Chang, Lin Zhang and Peng-Tao Xu contributed equally to this study.

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*Correspondence to: Dr. Zhi-Bin Yu, Department of Aerospace Physiology, Fourth Military Medical University, 169# Changlexi Road, Xi'an 710032, China. E-mail: yuzhib@fmmu.edu.cn

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al., 2009b]. The extracellular high glucose activates calpain-1 via the NADPH oxidase-dependent pathway and is associated with activation of L-type calcium channels and ryanodine receptors. The activated calpain-1 induces cardiomyocyte apoptosis through down-regulation of the Na^+/K^+ ATPase activity [Li et al., 2009b]. Calpain activation within the myocardium has been directly associated with IR-induced cardiomyocyte apoptosis. Indeed, the activated calpain(s) can induce cardiomyocyte apoptosis via several mechanisms. First, calpain cleaves a host of myocardial proteins, including structural (desmin) [Hikoso et al., 2007], contractile (troponin I and T subunits) [Gorza et al., 1997], and Ca^{2+}-handling proteins (L-type Ca^{2+} channels and phospholamban) [Singh et al., 2004], promoting their degradation by the proteasome [Goll et al., 2003]. Secondly, calpain cleaves and activates the proapoptotic factors BH3 interacting domain death agonist (BID) and caspase-12, stimulating caspase-3 activation and apoptosis [Chen et al., 2001; Bajaj and Sharma, 2006]. On the contrary, exercise can prevent IR-induced calpain activation in the myocardium to show cardioprotection against cardiomyocyte apoptosis [French et al., 2008]. Calpeptin, a pharmacological inhibitor of calpain, attenuates calpain activation and cardiomyocyte apoptosis in the pressure-overloaded feline myocardium [Mani et al., 2008]. Taurine prevents cardiomyocyte apoptosis by inhibiting NADPH oxidase-mediated calpain activation [Li et al., 2009a]. Over-expression of calpastatin inhibits calpain activation and cardiomyocyte apoptosis during the lipopolysaccharide stimulation [Li et al., 2009c]. These investigations on the mechanisms of cardiomyocyte apoptosis mainly focus on calpain-1 activation in the heart. Neuron and islet beta-cell apoptosis also involves calpain-2 activation [Tremper-Wells and Vallano, 2005; Huang et al., 2010], but the effect of calpain-2 on cardiomyocyte apoptosis has not been elucidated.

Therefore, the purpose of the present study was to test the hypothesis that a high dose of catecholamine may activate calpain-2 and further induce cardiomyocyte apoptosis during return from 4 weeks of tail-suspension.

MATERIALS AND METHODS

TAIL-SUSPENDED RAT MODEL

Healthy male Sprague–Dawley rats weighing 220 ± 10 g were used. Rats were randomly divided into the control group (CON), tail-suspended group (SUS) and recovery group (SUS + R) which released tail-suspension for 1 day. Tail-suspension was performed using a Morey-Holton method for 4 weeks [Morey-Holton and Globus, 2002]. Isoproterenol (ISO; Sigma–Aldrich, St. Louis, MO) was dissolved in physiological saline with equal amounts of vitamin C. A half of rats in the control and tail-suspended groups were intraperitoneally injected once with physiological saline or ISO (5 mg/kg body weight), respectively, before 1 day of experimental termination. The rats in the recovery group were administrated once with physiological saline, propranolol (10 mg/kg BW; Sigma–Aldrich) or PD150606 (PD; 10 mg/kg BW; Sigma–Aldrich), respectively, by intraperitoneal injection before 30 min of releasing suspension. All rats were housed in a $20 \pm 2^{\circ}C$ environment with a 12:12 h light-dark cycle, and were fed rat chow and water ad libitum. Care was taken to protect the tail tissue, and the movement of the rat was not restricted during the procedure. All animal procedures were approved by the Animal Care and Use Committee at the Fourth Military Medical University.

TUNEL DETECTION OF APOPTOTIC NUCLEI IN THE MYOCARDIUM

The heart was infused retrogradely, via aorta cannulation, with 20 ml of phosphate-buffered saline (PBS), followed by 40 ml of 4% paraformaldehyde in PBS for approximately 10 min. The heart was then fixed overnight. After paraffin embedding, sections were fixed to glass slides. The 5-μm-thick sections were deparaffinized by washing in xylene and a descending ethanol series. The sections were incubated with 8 μg/ml proteinase K for 5 min at room temperature. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using dUTP-FITC (FragEL™ DNA Fragmentation Detection Kit, Calbiochem, Darmstadt, Germany) was carried out according to the manufacturer's protocols. To specifically identify apoptotic cardiomyocytes, sections were concomitantly stained with a rabbit anti-desmin antibody (1:50, Cell Signaling Technology, Inc. (CST), Danvers, MA), followed by incubation with a tetramethyl-rhodamine-labeled (TRITC) secondary antibody (1:400; Molecular Probes, Eugene, OR) for 1 h at room temperature. Tissue sections were also counterstained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Sections were examined at a 60 × water objective using a laser-scanning confocal microscope (Olympus FV1000; Olympus Co., Ltd, Tokyo, Japan) equipped with the FV10-ASW system. Ten random fields per section were examined in a blinded fashion.

ISOLATION OF NUCLEI

Rat cardiac nuclei were isolated according to a previously described method [Boivin et al., 2003]. Briefly, rat hearts were homogenized (Polytron, 11,000 rpm; 2 × 10 s) in the homogenate buffer containing (in mM) 10 K-HEPES (pH 7.9), 1.5 $MgCl_2$, 10 KCl, 1 DTT, 0.2 Na_3VO_4, and 1 × Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged for 15 min at 500*g* and the supernatants were diluted 1:1 with the homogenate buffer, incubated for 10 min on ice, and centrifuged for 15 min at 2,000*g*. The resulting supernatant was discarded and the pellet was resuspended in buffer A (0.3 M K-HEPES, pH 7.9, 1.5 M KCl, 0.03 M $MgCl_2$, 0.2 mM Na_3VO_4, and 1 × Protease Inhibitor Cocktail), incubated on ice for 10 min, and centrifuged for 15 min at 2,000*g*. The supernatant was removed and the pellet (the enriched nuclear fraction) was resuspended in buffer B (20 mM Na-HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM DTT, 0.2 mM Na_3VO_4, and 1 × Protease Inhibitor Cocktail) for the zymography analysis or in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 1% SDS for Western blot. All above steps were carried out on ice or at 4°C.

CALPAIN ACTIVITY ASSAY

Calpain activity was measured by a casein zymography method described elsewhere [Raser et al., 1995]. The 0.2% alkali-denatured casein (Sigma–Aldrich) was co-polymerized in 10% mini-gels (0.75 mm thickness). Gels were pre-run with the zymography running buffer containing (in mM) 25 Tris–HCl, pH 8.3, 192 glycine,

1 EGTA, and 1 dithiothreitol (DTT) for 15 min at 4°C. Each sample and purified calpain-1 or calpain-2 (50, 100, 200, 400, 800, and 1,000 ng; Calbiochem) were subsequently loaded and run. Gels were incubated in the zymography development buffer containing 20 mM Tris (pH 7.3), 10 mM DTT, and 5 mM calcium at room temperature overnight. Gels were stained with Coomassie brilliant blue R-250. After destaining, calpain activity developed as clear bands against a dark background indicating proteolysis of casein and quantified with the NIH Image J software (available at http://rsbweb.nih.gov/ij/download.html). The protein concentration in the muscle homogenate was determined by a Bradford method, using bovine albumin fraction V (Sigma–Aldrich) as a standard.

WESTERN BLOT ANALYSIS

As described previously, total protein was extracted from the left ventricular myocardium of the rat by homogenization in SDS–PAGE sample buffer containing 1% SDS. The myocardial protein extracts were resolved by SDS–PAGE using Laemmli gels. Ten percent gel was used for the examination of calpain-1, calpain-2, and calpastatin; and 12% gel was used for the examination of lamin B1 and GAPDH. After electrophoresis, proteins were electrically transferred to nitrocellulose membrane (0.45 μm pore size) using a Bio-Rad semi-dry transfer apparatus. The blotted nitrocellulose membranes were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris–HCl, pH 7.5) and incubated with a rabbit polyclonal anti-calpain-1 large subunit (1:1,000; CST), rabbit polyclonal anti-calpain-2 large subunit (1:1,000; CST), rabbit polyclonal anti-calpastatin (1:1,000; CST), mouse monoclonal anti-lamin B1 (1:1,000), or mouse monoclonal anti-GAPDH (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBS containing 0.1% BSA at 4°C overnight. The membranes were incubated with IRDye 680CW goat-anti mouse or IRDye 800CW goat-anti rabbit secondary antibodies (1:10,000) for 90 min at room temperature, and visualized using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE). Quantification analysis of blots was performed with the NIH Image J software.

ISOLATION AND CULTURE OF ADULT RAT CARDIOMYOCYTES

Cardiomyocytes were isolated from the hearts of control and tail-suspended rats using a previously described technique [Nagata et al., 1998]. The heart was rapidly excised and immediately immersed in Ca^{2+}-free Joklik solution (Sigma–Aldrich) containing 10 mM HEPES and 10 mM $NaHCO_3$. The heart was perfused in the Langendorff mode with non-circulating Joklik solution at a constant flow of 8 ml/min for 5 min and then perfused with the circulating digestion Joklik solution containing 0.08% collagenase I (Sigma–Aldrich) plus 0.1% BSA for 30 min. Finally, the digestion Joklik solution was washed out with Joklik solution for 5 min. The ventricular myocardium was cut into small pieces and gently agitated. The cardiomyocytes were filtered through mesh screens, centrifuged for 3 min at 400*g*, and resuspended in a warm Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corporation, Carlsbad, CA) containing 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 0.1 mM insulin, 100 U/ml penicillin–streptomycin and 0.1% BSA. Cardiomyocytes were plated on culture chamber slides coated with 10 μg/ml laminin (Sigma–Aldrich) at $3–5 \times 10^4$ cells per well and incubated in a CO_2 incubator at 37°C. After settling on the slide, cardiomyocytes were cultured with or without 10 nM ISO for 24 h, or pre-incubated with 10 μM PD for 30 min, and subsequently treated with 10 nM ISO plus 10 μM PD for 24 h.

SMALL RNA INTERFERENCE

The rat calpain-1 and calpain-2 small interfering RNA (siRNA) and scrambled siRNA as a negative control were purchased from Santa Cruz Biotechnology. Adult rat cardiomyocytes were transfected with calpain-1, calpain-2 and scrambled siRNA oligonucleotides by using TransMessenger Transfection Reagent (Qiagen) according to the manufacturer's instructions. Briefly, we mixed 3 μl Enhancer, 84 μl Buffer ECR, 4.5 μl siRNA (10 mM), 6 μl TransMessenger, and 220 μl DMEM without serum/antibiotics for each well (12-well plates). After incubation with the siRNA mixture for 4 h, the medium was changed with a fresh DMEM. The cells were then incubated for 24 h before treatment with 10 nM ISO.

IMMUNOFLUORESCENT CYTOCHEMISTRY AND CONFOCAL ANALYSIS

Adult rat cardiomyocytes were fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were permeabilized in 0.1% Triton X-100/PBS for 30 min, blocked with 1% BSA in PBS for 60 min at room temperature, and then incubated with mouse monoclonal anti-PLB (1:100; CST), mouse monoclonal anti-phosphorylated PLB at serine-16 residue (Phospho-Ser16; 1:100; CST), anti-calpain-1 (1:100), anti-calpain-2 (1:25), or anti-calpastatin antibody (1:50) at 4°C overnight. The slides were rinsed twice in PBS and incubated with TRITC-labeled goat anti-rabbit IgG or Alexa Fluor 488-labeled goat anti-mouse IgG (1:400; Molecular Probes) for 60 min. Staining was observed using a laser-scanning confocal microscope equipped with the FV10-ASW system (Olympus FV1000). The TRITC- and Alexa Fluor 488-labeled signals were visualized at 555 and 519 nm. Images were acquired at a 60 × water objective. Optical densitometry analysis of nuclear calpain-2 was performed using Olympus Fluoview image analysis software (Olympus Co., Ltd). Apoptotic nuclei were identified by the Hoechst33258 staining. The percentage of apoptotic myocytes was calculated from the ratio of apoptotic cells to total cells in each field.

INTRACELLULAR CALCIUM MEASUREMENTS

Cardiomyocytes attached to a laminin-coated glass slide were loaded with the fluorescent Ca^{2+} indicator fluo-3/AM for 30 min at 37°C and then perfused with Tyrode's solution containing (in mM) 132 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 5.0 sodium pyruvate, 10 HEPES, and 10 D-glucose, pH 7.4 at a flow rate of 0.2 ml/min at room temperature. Electric field stimulus (rectangle wave, 15 V, 5 ms, and 0.5 Hz) was administered by the stimulator. The free Ca^{2+} concentration of cardiomyocytes in the cytoplasm and the nucleus was measured using fluorescence laser scanning confocal microscopy (Olympus FV1000) before and after 10 nM ISO administration. Fluo-3 was excited at 488 nm and fluorescence was measured at wavelengths >515 nm. Images were acquired at 10 ms intervals. Further image analysis was carried out using Olympus Fluoview image analysis software (Olympus).

STATISTICAL ANALYSIS

Data are presented as means ± SE. Data for cultured cardiomyocytes are mean values of three different experiments. Differences between two groups were compared by unpaired Student's *t*-test. For multi-group comparisons, 1-way ANOVA followed by Tukey post hoc test was performed. A value of $P < 0.05$ was considered statistically significant.

RESULTS

ISO STIMULATION IN 4-WEEK TAIL-SUSPENDED OR 1-DAY RECOVERY RATS INCREASES CARDIOMYOCYTE APOPTOSIS

To explore the effect of 1-day recovery on cardiomyocyte apoptosis and its possible trigger, 4-week tail-suspended rats were released from the suspension for 1 day or intraperitoneally injected with ISO. The number of TUNEL-positive myonuclei of left ventricular myocardium in tail-suspended rats was similar to that in the synchronous control (Fig. 1A,B). Apoptotic rate of cardiomyocytes increased significantly in tail-suspended rats after ISO stimulation, but not in the synchronous control. One-day recovery increased cardiomyocyte apoptosis which was blocked by propranolol, a blocker of β-adrenergic receptor or PD150606, an inhibitor of calpain-1 and -2 (Fig. 1C). Therefore, ISO may be a trigger of cardiomyocyte apoptosis and calpains may also be involved in cardiomyocyte apoptosis in the recovery group.

TOTAL CALPAIN-2 ACTIVITY INCREASES AND THE ACTIVATED CALPAIN-2 TRANSLOCATES TO THE NUCLEI OF CARDIOMYOCYTES IN TAIL-SUSPENDED RATS

To elucidate the isoform of calpain involved in cardiomyocyte apoptosis, the activity and expression of calpain-1 and -2 were observed in the myocardium from tail-suspended and control rats. Calpain-1 and -2 activities were measured by the casein zymography (Fig. 2A). Calpain-2 activities in the myocardium

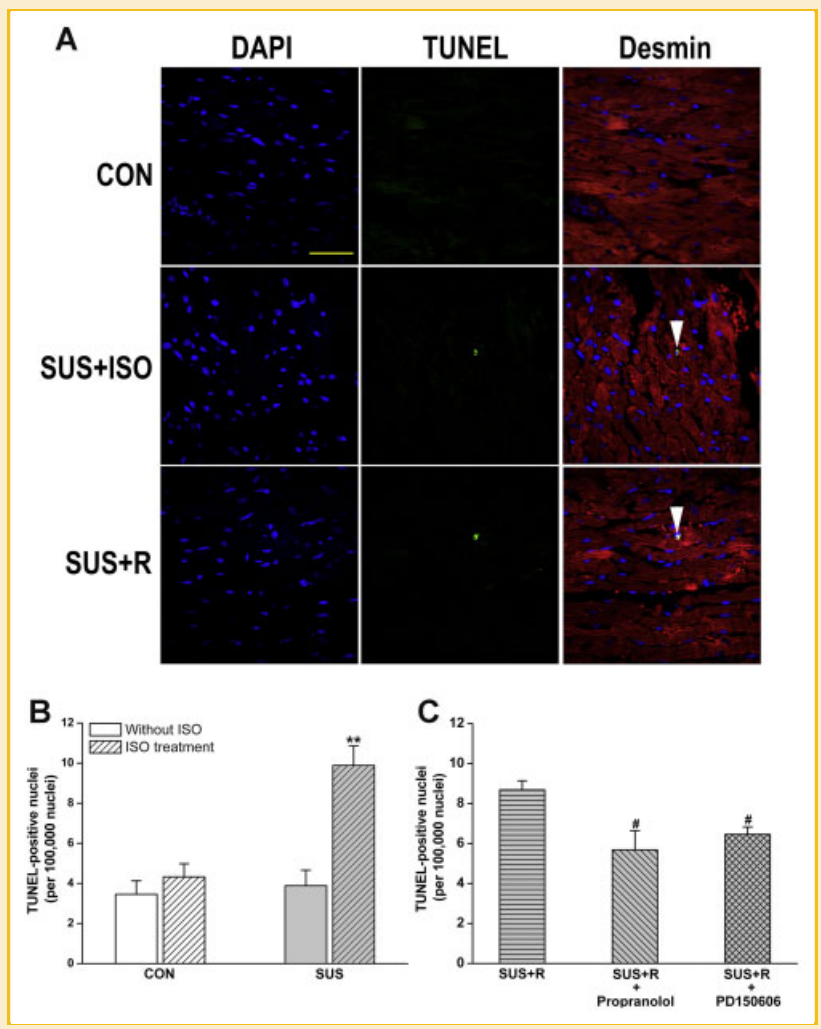

Fig. 1. Apoptotic rates of cardiomyocytes increase in left ventricles of 4-week tail-suspended rats after ISO stimulation and 1-day recovery. A: DAPI-labeled nuclei, FITC-labeled TUNEL-positive nuclei, and desmin-labeled myocardium; arrowheads indicate TUNEL-positive myonuclei. Scale Bar = 50 μm. B,C: The percentage of TUNEL-positive myonuclei was quantified on myocardial sections from the indicated treatments. The results from examination of at least 100,000 nuclei per animal are shown. Values are mean ± SE; n = 6 rat hearts in each group. $^{**}P < 0.01$ versus SUS without ISO treatment, $^{\#}P < 0.05$ versus recovery group (SUS + R).

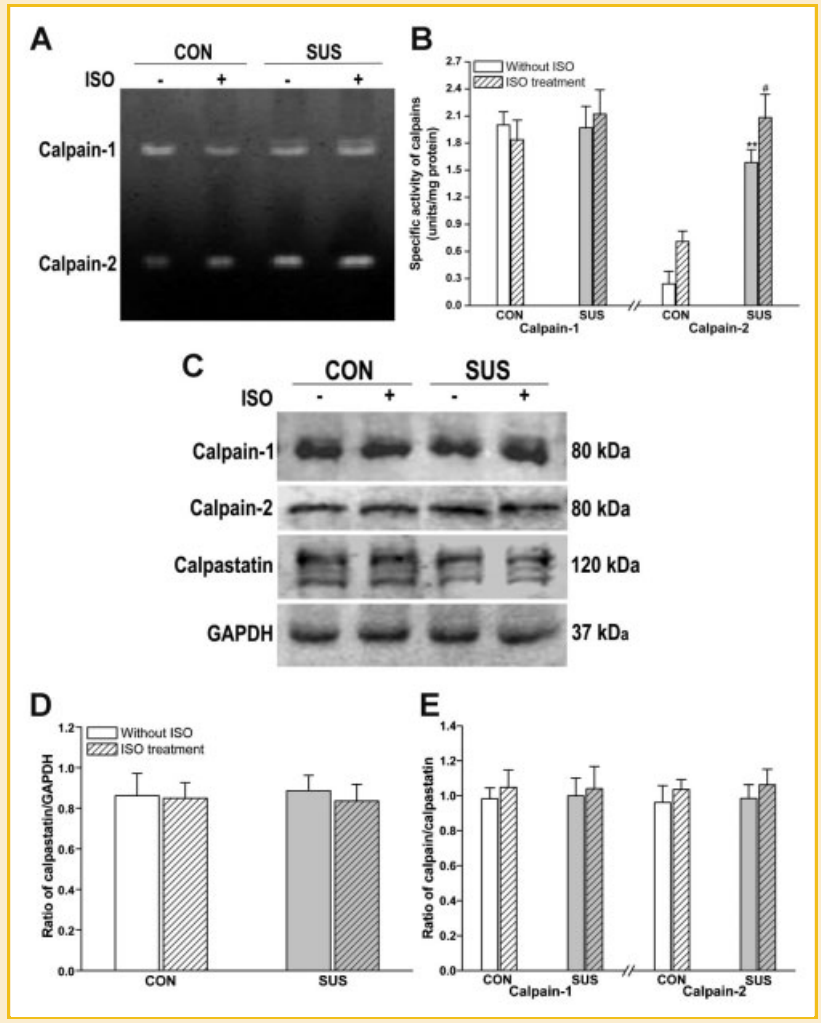

Fig. 2. Activity and expression of cytoplasmic calpain-1, -2, and calpastatin in the left ventricle of 4-week tail-suspended rats. A: Representative casein zymography. B: Activity of calpain-2 increased in the left ventricle of 4-week tail-suspended rats, whereas activity of calpain-1 was unchanged. Values are mean ± SE; n = 6 rat hearts in each group. $^{**}P < 0.01$ versus CON without ISO treatment. $^{\#}P < 0.01$ versus SUS without ISO treatment. C: Representative immunoblots of calpain-1, calpain-2, and calpastatin in the left ventricle. D,E: Calpastatin/GAPDH, calpain-1/calpastatin, and calpain-2/calpastatin ratios were unchanged in the control and 4-week tail-suspended rats with and without 10 nM ISO treatment. Values are mean ± SE; n = 6 rat hearts in each group.

showed a marked increase in tail-suspended rats compared with the control, whereas there was no significant difference in calpain-1 activity between the control and tail-suspended groups. ISO treatment did not affect calpain-1 activities in the myocardium from the control and tail-suspended rats, but calpain-2 activities increased in tail-suspended rats (Fig. 2B).

Expression of calpastatin in the myocardium was unchanged in tail-suspended and control groups (Fig. 2C,D). Calpain-1/calpastatin and calpain-2/calpastatin ratios in the myocardium were also unchanged in the tail-suspended group compared with the control group (Fig. 2E). ISO stimulation did not affect expression of calpain-1, -2, and calpastatin in tail-suspended and control groups.

Calpain-2 activity, but not calpain-1, increased in isolated nuclei of cardiomyocytes in the tail-suspended group (Fig. 3A,B). The isolated nuclei of cardiomyocytes in tail-suspended rats showed a significant increase in expression of calpain-2, but not in expression of calpain-1 (Fig. 3C,D). The activity and expression of calpain-1 were unaltered, whereas the activity and expression of calpain-2 were increased under ISO treatment in the isolated nuclei of cardiomyocytes from tail-suspended rats. The nuclear calpastatin was not measurable by Western blot in both tail-suspended and control groups.

PD150606 AND CALPAIN-2 KNOCKDOWN INHIBIT ISO-INDUCED CARDIOMYOCYTE APOPTOSIS AND NUCLEAR TRANSLOCATION OF CALPAIN-2 IN TAIL-SUSPENDED RATS

To further confirm the effects of ISO stimulation on nuclear translocation of calpain-2 and cardiomyocyte apoptosis, the isolated cardiomyocytes were observed by an immunofluorescent cytochemical technique. There were no differences in apoptotic rates of cardiomyocytes between the control groups with and without ISO treatment (Fig. 4A,B). ISO stimulation induced a higher level of cardiomyocyte apoptosis in the tail-suspended group, but PD150606

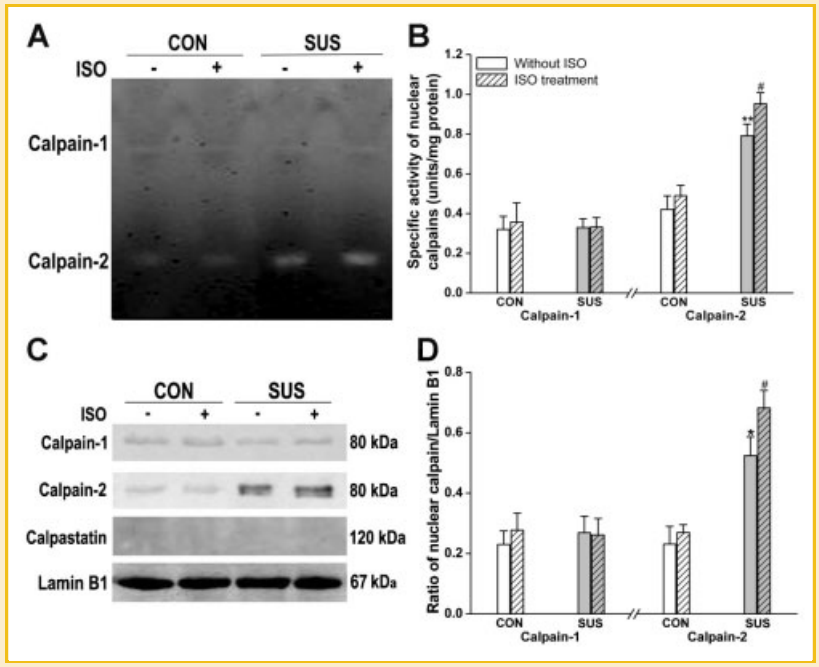

Fig. 3. Activity and expression of nuclear calpain-1, -2, and calpastatin in the left ventricle of 4-week tail-suspended rats. A: Representative casein zymography. B: Activity of calpain-2 increased in the isolated cardiomyocyte nuclei of 4-week tail-suspended rats, whereas activity of calpain-1 was unchanged. Values are mean ± SE; n = 6 rat hearts in each group. $^{**}P < 0.01$ versus CON without ISO treatment. $^{\#}P < 0.01$ versus SUS without ISO treatment. C: Representative immunoblots of calpain-1, calpain-2, and calpastatin in the isolated nuclei of cardiomyocytes. D: Calpain-2/lamin B1 ratio in nuclei increased in 4-week tail-suspended rats. Values are mean ± SE; n = 6 rat hearts in each group. $^{*}P < 0.01$ versus CON without ISO treatment. $^{\#}P < 0.01$ versus SUS without ISO treatment.

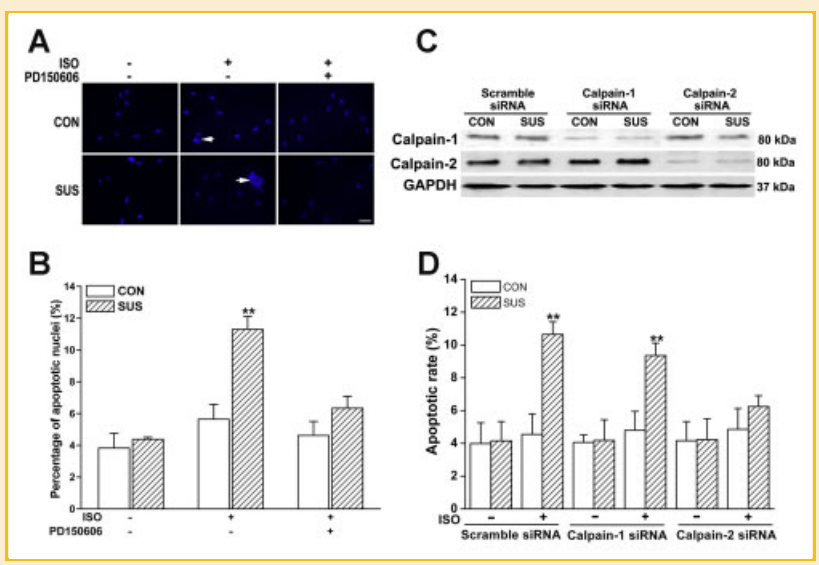

Fig. 4. PD150606 or calpain-2 down-regulation inhibits ISO-induced cardiomyocyte apoptosis in 4-week tail-suspension rats. A: Representative apoptotic nuclei of adult cardiomyocyte by Hoechst33258 staining. Arrows indicate apoptotic myonuclei. Scale Bar = 25 μm. B: Bar graph depicting the changes in apoptotic rates of isolated cardiomyocytes from control and 4-week tail-suspended rats with and without 10 nM ISO stimulation. Values are mean ± SE; n = 3 independent experiments. At least 500 nuclei of cardiomyocytes were counted in each group. $^{**}P < 0.05$ versus synchronous CON with ISO treatment. C: Representative immunoblots of calpain-1 and calpain-2 in the isolated cardiomyocytes transfected by scramble, calpain-1, and calpain-2 siRNA, respectively. D: Apoptotic rates of cardiomyocytes transfected by scramble, calpain-1, and calpain-2 siRNA, respectively, with or without ISO treatment. Values are mean ± SE; n = 3 independent experiments. $^{**}P < 0.05$ versus synchronous CON with ISO treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

reduced ISO-induced cardiomyocyte apoptosis to the level observed without ISO treatment in the tail-suspended group (Fig. 4B).

The scramble siRNA unaltered calpain-1 and -2 expressions in cardiomyocytes from the control and tail-suspended rats. Calpain-1 siRNA significantly reduced calpain-1 but not calpain-2 expression (Fig. 4C). Similarly, calpain-2 siRNA downregulated calpain-2 but not calpain-1 expression (Fig. 4C). ISO treatment significantly increased apoptotic rates of cardiomyocytes transefected with scramble or calpain-1 siRNA in the tail-suspended group. In contrast, apoptotic rate of calpain-2 siRNA-transfected cardiomyocytes was not increased during ISO treatment in the tail-suspended group (Fig. 4D).

Confocal microscope immunofluorescence images indicated that calpain-1, calpain-2, and calpastatin were distributed in the cytoplasm of cardiomyocytes (Fig. 5A). Calpain-1 and -2 showed a small amount of nuclear accumulation in control cardiomyocytes. The nuclear accumulation of calpain-2 in cardiomyocytes was greater in the tail-suspended group than in the control, but the nuclear accumulation of calpain-1 was unaltered in both groups. ISO treatment promoted the nuclear translocation of calpain-2 in tail-suspended rat cardiomyocytes while in contrast, PD150606 reduced ISO-induced nuclear translocation of calpain-2 (Fig. 5B).

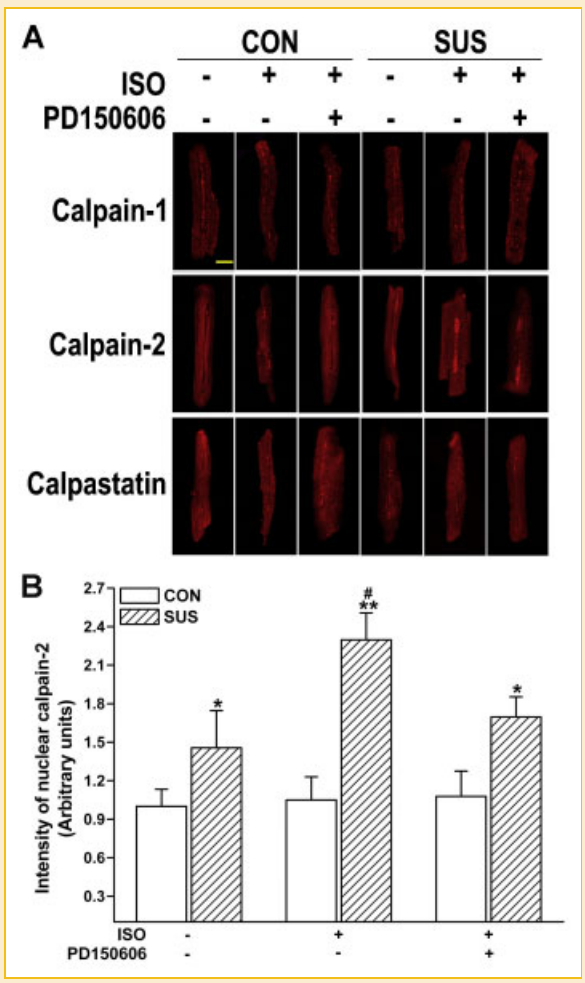

Fig. 5. Distribution of calpain-1, calpain-2, and calpastatin in cardiomyocytes. A: Representative immunofluorescence images of cardiomyocytes. Cultured cardiomyocytes were exposed to 10 nM ISO for 24 h, or pre-incubated with 10 μM PD150606 for 30 min, followed by treatment of 10 nM ISO plus 10 μM PD for 24 h. Scale bar = 30 μm. B: The fluorescence intensity of calpain-2 in nuclei was analyzed in three independent experiments. Values are mean ± SE. At least 200 cardiomyocytes were analyzed in each group. $^{*}P < 0.05$ or $^{**}P < 0.01$ versus CON without ISO treatment. $^{\#}P < 0.05$ versus SUS without ISO treatment.

ISO STIMULATION INCREASES THE PHOSPHORYLATED PLB OF THE NUCLEAR ENVELOPE AND SUBSEQUENTLY ELEVATES INTRANUCLEAR Ca^{2+} TRANSIENT OF CARDIOMYOCYTES IN TAIL-SUSPENDED RATS

To test the hypothesis that an ISO-induced increase in the intranuclear Ca^{2+} activates nuclear calpain-2 and subsequently triggers cardiomyocyte apoptosis, the levels of the phosphorylated PLB of the nuclear envelope and intranuclear Ca^{2+} transients were measured in tail-suspended and control rats. Figure 6 shows representative confocal images of control (left) and tail-suspended (right) cardiomyocytes in the absence or the presence of ISO. PLB of

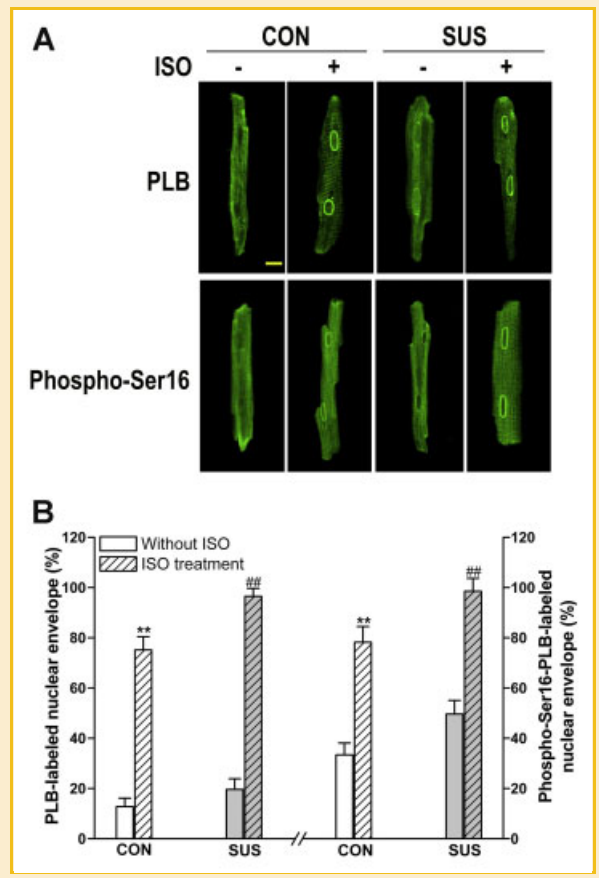

Fig. 6. Phosphorylated phospholamban (PLB) at serine-16 residue increases in the nuclear envelope of cardiomyocytes after 10 nM ISO stimulation. A: Representative immunofluorescence images of cardiomyocytes. Cultured cardiomyocytes were exposed to 10 nM ISO for 24 h. Scale bar = 30 μm. B: PLB-labeled and phosphorylated Ser16-PLB-labeled nuclear envelope was counted among 300 cardiomyocytes in three independent experiments. Values are mean ± SE. $^{**}P < 0.01$ versus CON without ISO treatment or tail-suspended group without ISO treatment. $^{\#\#}P < 0.01$ versus CON with ISO treatment.

the nuclear envelope was similar between tail-suspended and control rats before ISO administration. After ISO stimulation, PLB accumulation at the nuclear envelope of cardiomyocytes was significantly increased in control and tail-suspended groups, while PLB accumulation at the nuclear envelope in tail-suspended rats was more than that in the control rats. The ISO-induced increase in the phosphorylated PLB at the nuclear envelope in the tail-suspended group was also more than that in the control. These data indicate that the accumulation of PLB at the nuclear envelope is the phosphorylated PLB at the serine-16 residue.

Figure 7A illustrates a series of two-dimensional confocal images of a cardiomyocyte during an electrically stimulated Ca^{2+} transient before and after ISO treatment. The myocyte exhibited quite uniform resting fluorescence, indicating that Fluo-3 was distributed mainly in the cytoplasm. Following stimulation, intracellular Ca^{2+} fluorescence started to increase at the one end and then rapidly propagated to the other end of the cardiomyocyte. The increase in nuclear Ca^{2+} fluorescence was slightly delayed compared with the cytoplasmic Ca^{2+} fluorescence. The resting and peak Ca^{2+} fluorescence intensity of cardiomyocytes in the tail-suspended group was not different from those in the control. ISO stimulation had no apparent effect on resting Ca^{2+} fluorescence intensity in the tail-suspended and control groups, but ISO stimulation did increase cytoplasmic peak Ca^{2+} fluorescence intensity in both tail-suspended and control groups. ISO treatment also significantly increased peak intranuclear Ca^{2+} fluorescence intensity in the tail-suspended group compared with the control (Fig. 7B).

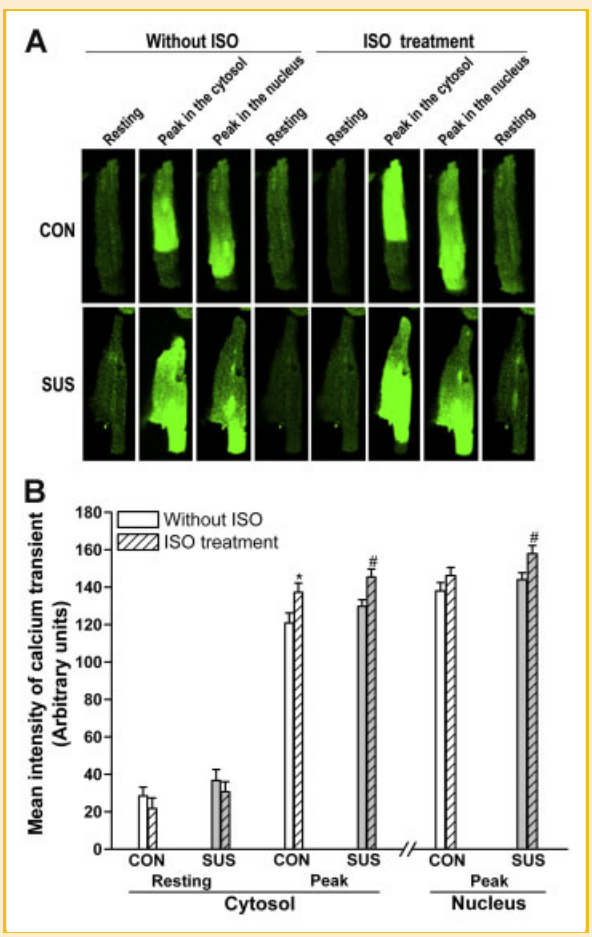

Fig. 7. ISO stimulation increases cytoplasmic and intranuclear peak Ca^{2+} transient of cardiomyocytes in 4-week tail-suspended rats. A: Representative fluorescence images of cardiomyocytes loaded by fluo-3/AM for 30 min. B: Bar graph depicting the changes in the mean intensity of cytoplasmic resting and peak Ca^{2+} and intranuclear peak Ca^{2+} of cardiomyocytes before and after 10 nM ISO treatment. Ten cardiomyocytes were analyzed in each group. Values are mean ± SE. $^{*}P < 0.05$ versus CON without ISO treatment. $^{\#}P < 0.05$ versus SUS without ISO treatment.

DISCUSSION

ENHANCED NUCLEAR TRANSLOCATION OF CALPAIN-2 INCREASES THE PROPENSITY TOWARD CARDIOMYOCYTE APOPTOSIS IN 4-WEEK TAIL-SUSPENDED RATS

The adaptation of a bed rest position with the head-down by 6 degrees can simulate the effects of microgravity on the cardiovascular system in humans. Long-term bed rest induces dramatic reductions in maximal stroke volume and cardiac output [Convertino et al., 1997a]. Underlying physiological mechanisms working to reduce stroke volume appear to function through a reduction in the preload associated with less circulating blood volume and depressed cardiac contractility [Yu et al., 2001]. Lower responsiveness of vessels to catecholamine leads to a reduction in peripheral vascular resistance in spaceflight astronauts and simulated weightlessness rats. Although the blood pressure is unchanged, the cardiovascular system is under the hypokinetic status. The hypokinetic status of the heart may increase the activation of calpains [Razeghi et al., 2007]. In 14-day suspended rats, the amplitude of twitch Ca^{2+} transient is increased without significant modifications of basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of cardiomyocytes [Halet et al., 1999]. The increase in twitch peak $[Ca^{2+}]_i$ may activate calpain-1 and/or calpain-2 in cardiomyocytes. Troponin I and T subunits are regulatory proteins in the thin filament and sensitive to calpain degradation in the IR heart [Barta et al., 2005]. The increased degradation in the N-terminus of cardiac troponin I also indicates an enhanced activation of calpain in the heart of the tail-suspended rat [Yu et al., 2001]. In the present study, we found that calpain-2 activity was significantly increased, but calpain-1 activity only showed an increasing trend in the tail-suspended rat heart. The tail-suspension also induced nuclear translocation of calpain-2. Despite the activation and nuclear translocation of calpain-2, the apoptotic rate of cardiomyocytes did not increase in 4-week tail-suspended rats. Therefore, the enhanced nuclear translocation of calpain-2 may increase susceptibility to apoptosis in cardiomyocytes of 4-week tail-suspended rat.

The expression of troponin I and lamin B1 was detected in the homogenate and isolated nuclei of myocardium (data not shown). The expression of lamin B1 in homogenate was less 10% than that in isolated nuclei. The isolated nuclei contaminated a little TnI (<5%). Therefore, the purity of isolated nuclei was more than 95%. The confocal images of cardiomyocytes against calpain-1 in Figure 5A did not show any calpain-1 in the nuclei. Thus, the slight expression of calpain-1 in isolated nuclei may be a contamination of cytosolic calpain-1.

ISO PROMOTES AN INCREASE IN CYTOPLASMIC AND INTRANUCLEAR Ca^{2+} CONCENTRATION IN CARDIOMYOCYTES IN TAIL-SUSPENDED RATS

There is a transient rise in cytoplasmic Ca^{2+} during each heart beat caused by a Ca^{2+}-induced Ca^{2+} release mechanism in cardiomyocytes. ISO regulates cytoplasmic Ca^{2+} transient through the protein kinase A (PKA) signal pathway. Phosphorylation of phospholamban (PLB) at the serine-16 and/or thronine-17 residues by PKA ameliorates the inhibition to Ca^{2+}-ATPase of sarcoplasmic reticulum (SERCA) and subsequently increases Ca^{2+} uptake by SERCA. The amplitude of Ca^{2+} transient increases at the next heart beat [Periasamy et al., 2008]. Recent research has indicated that there is an independent intranuclear Ca^{2+} handling system, named the nucleoplasmic reticulum in cardiomyocytes and skeletal muscle fibers [Marius et al., 2006; Luo et al., 2008]. The nucleoplasmic reticulum is a nuclear Ca^{2+} storage organelle consisting of the endo/sarcoplasmic reticulum and the nuclear envelope. This specialized organelle also expresses functional inositol 1,4,5-trisphosphate receptors (InsP3R), ryanodine receptors (RyR), and PLB [Brette et al., 2004; Marius et al., 2006]. Therefore, ISO activates PKA, subsequently phosphorylates PLB, and regulates intranuclear Ca^{2+} transient in the same manner as regulating cytoplasmic Ca^{2+} transient by the sarcoplasmic reticulum [Marius et al., 2006]. In the current study, since the sensitivity of the β-adrenergic receptor increases in tail-suspended rats [Convertino et al., 1997b], the accumulation of phosphorylated PLB at the nuclear envelope of cardiomyocytes was greater in tail-suspended rats than in control rats. Thus, the amplitude of nuclear Ca^{2+} transient was higher in tail-suspended rats under ISO stimulation. The intranuclear Ca^{2+} signaling is an important regulator of gene transcription, apoptosis, and cardiac function [George et al., 2007; Guatimosim et al., 2008]. The alternatively spliced variants of the human cardiac RyR channel can modulate intracellular and nuclear Ca^{2+} signaling and are key determinants of cardiomyocyte apoptotic susceptibility [George et al., 2007]. Therefore, ISO-induced larger nuclear Ca^{2+} transient of cardiomyocytes in tail-suspended rats may be related to cardiomyocyte apoptosis.

LARGE INTRANUCLEAR Ca^{2+} FURTHER ACTIVATES NUCLEAR CALPAIN-2 TRANSLOCATED FROM THE CYTOPLASM AND SUBSEQUENTLY INDUCES CARDIOMYOCYTE APOPTOSIS IN TAIL-SUSPENDED RATS

The activated calpains translocate to target proteins where induce proteolysis. The nucleus is one of translocation sites of calpain-2 [Goll et al., 2003]. ISO further activated calpain-2 in cardiomyocytes of tail-suspended rats, and then led more nuclear translocation of calpain-2. Based on the nuclear translocation of calpain-2, the higher amplitude of Ca^{2+} transient may induce cardiomyocyte apoptosis in tail-suspended rats during ISO stimulation. Calpastatin, an endogenous inhibitor of calpains, did not transit to the nuclei in cardiomyocytes of tail-suspended rats. Although the ratios of calpain-1 or -2 to calpastatin were not different between the control and tail-suspended rats, the activity of nuclear calpain-2 lacked the inhibition of calpastatin increased in tail-suspended rats. However, the increased calpain-2 activity did not reach a level sufficient to trigger apoptosis in the cardiomyocytes of tail-suspended rats. The larger nuclear Ca^{2+} concentration further enhanced the calpain-2 activity under ISO stimulation in tail-suspended rats. Subsequently, the higher activation of calpain-2 mediated cardiomyocyte apoptosis. The underlying mechanism of nuclear calpain-2 induced-apoptosis remains elusive. The nuclear calpain-2 may cleave the proteins related to apoptosis [Chen et al., 2001; Bajaj and Sharma, 2006].

PD150606, a specific inhibitor of calpain-1 and -2, not only attenuated cardiomyocyte apoptosis in ISO-treated tail-suspended rats, but also in rats recovering from 4-week tail-suspension. PD150606 efficiently inhibited the nuclear translocation of activated calpain-2 during ISO stimulation. Especially, calpain-2 knockdown by siRNA blocked cardiomyocyte apoptosis during ISO stimulation in tail-suspended rats. Therefore, the higher level of calpain-2 activation plays an important role on cardiomyocyte apoptosis in tail-suspended rats. When the tail-suspension is removed in the rat, an increase in catecholamine release occurs due to the reduced blood volume. The enhanced activation of β-adrenergic receptor will trigger cardiomyocyte apoptosis based on moderate activation and nuclear translocation of calpain-2 in 1-day recovery rats. Thus calpain-2 is a molecular target for preventing the heart against cardiomyocyte apoptosis and cardiac dysfunction in the tail-suspended rat. Until now, there is no evidence for cardiac damage that occurs in astronauts immediately after returning from space flight. This study suggests that the serum concentrations of cardiac LDH isoforms, troponin I and T should be investigated in the future.

In summary, long-term tail-suspension induced a cephalic redistribution of fluid and subsequently led to the unloading of the heart due to the reduced blood volume. Calpain-2 was activated and translocated to the nucleus in the unloading heart. The recovery from 4-week tail-suspension might result in a high dose of catecholamine. ISO stimulation increased the phosphorylated PLB of the nuclear envelope and elevated the intranuclear Ca^{2+} peak of cardiomyocytes. Increasing in intranuclear Ca^{2+} levels further activated nuclear calpain-2 without the endogenous inhibitor of calpastatin and then induced cardiomyocyte apoptosis.

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