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ROS-Induced Nuclear Translocation of Calpain-2 Facilitates Cardiomyocyte Apoptosis in Tail-Suspended Rats

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ABSTRACT

Isoproterenol (ISO) induced nuclear translocation of calpain-2 which further increased susceptibility of cardiomyocyte apoptosis in tail-suspended rats. The underlying mechanisms remain elusive. In the present study, the results showed that ISO (10 nM) significantly elevated NADPH oxidases (NOXs) activity and NOXs-derived ROS productions which induced nuclear translocation of calpain-2 in cardiomyocytes of tail-suspended rats. In contrast, the inhibition of NADPH oxidase or cleavage of ROS not only reduced ROS productions, but also resisted nuclear translocation of calpain-2 and decreased ISO-induced apoptosis of cardiomyocyte in tail-suspended rats. ISO also increased the constitutive binding between calpain-2 and Ca^{2+}/calmodulin-dependent protein kinase II δ_B (CaMK II δ_B) in nuclei, concomitant with the promotion of CaMK II δ_B degradation and subsequent down-regulation of Bcl-2 mRNA expression and the ratio of Bcl-2 to Bax protein in tail-suspended rat cardiomyocytes. These effects of ISO on cardiomyocytes were abolished by a calpain inhibitor PD150606. Inhibition of calpain significantly reduced ISO-induced loss of the mitochondrial membrane potential, cytochrome c release into the cytoplasm, as well as the activation of caspase-3 and caspase-9 in mitochondrial apoptotic pathway. In summary, the above results suggest that ISO increased NOXs-derived ROS which activated nuclear translocation of calpain-2, subsequently nuclear calpain-2 degraded CaMK II δ_B which reduced the ratio of Bcl-2 to Bax, and finally the mitochondria apoptosis pathway was triggered in tail-suspended rat cardiomyocytes. Therefore, calpain-2 may represent a potentially therapeutic target for prevention of oxidative stress-associated cardiomyocyte apoptosis. J. Cell. Biochem. 116: 2258–2269, 2015.

KEY WORDS: CARDIOMYOCYTE; APOPTOSIS; CALPAIN-2; NUCLEAR TRANSLOCATION; NADPH OXIDASE

Calpains, calcium-dependent proteases, include ubiquitous and tissue-specific isoforms, the most common being calpain-1 and calpain-2, consisting of identical regulatory and distinct catalytic subunits that confer different sensitivities to Ca^{2+} [Goll et al., 1992]. In addition, calpastatin is an endogenous inhibitor of calpains [Goll et al., 2003]. Myocardium expresses moderate amounts of calpain-1, calpain-2 and calpastatin [Galvez et al., 2007]. The micromolar Ca^{2+}-activated calpain-1 and the millimolar Ca^{2+}-activated calpain-2 are implicated in apoptosis of cardiomyocytes in the diabetic and ischemia-reperfusion heart [Chen et al., 2001; Li et al., 2009b]. The nuclear translocation of calpain-2 increased susceptibility of cardiomyocyte apoptosis in tail-suspended rats [Chang et al., 2011]. Although intracellular Ca^{2+} concentration is considerably elevated in cardiomyocytes during isoproterenol (ISO) stimulation, but not reaches a millimolar level to activate calpain-2. Therefore, the mechanism of calpain-2 activation has not been fully elucidated in cardiomyocytes of tail-suspended rats.

Spaceflight induces an increase in oxidative stress and reduction in some blood antioxidants in astronauts [Schoenfeld et al., 2011;

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Mao et al., 2013]. The effects are more pronounced after long-term spaceflight [Li et al., 2014]. Most rodent studies also show the increased production of lipid peroxidation and decreased antioxidant enzyme activity postflight or after tail-suspension [Chowdhury and Soulsby, 2002; Lawler et al., 2003]. Increased reactive oxygen species (ROS) have been shown to induce calpain-2 activation in retinal photoreceptor cells [Sanvicens et al., 2004]. Excessive TRPM7 channel activity in HEK-293 cells causes oxidative stress, producing cell rounding and loss of adhesion mediated by activation of calpain-2 [Su et al., 2010]. Neuronal damage involves ROS production by NADPH oxidase 2 (NOX2), which, in turn, contributes to calpain activation [Guemez-Gamboa et al., 2011]. Taurine prevents cardiomyocyte apoptosis by inhibiting NOX-mediated calpain activation [Li et al., 2009a].

NOXs are a major source of ROS in the myocardium [Kumar and Jugdutt, 2003]. There are two isoforms of NOX expressed in the heart being NOX2 and NOX4 [Nabeebaccus et al., 2011]. Selective increase in β_1- and β_2-adrenoreceptor responsiveness in the heart is one of the most profound changes that occur in man during prolonged exposure to weightlessness environment in space [Convertino et al., 1997]. The sympathetic adrenergic system plays a central role in stress signaling and stress is often associated with increased production of ROS. Endogenously produced ROS contributes to the inotropic mechanism of β-adrenergic stimulation in mouse cardiomyocytes [Andersson et al., 2011]. NOX-mediated ROS production is implicated in α_1-adrenoreceptor-stimulated hypertrophy of adult rat ventricular myocytes [Xiao et al., 2002]. Transgenic activation of β_2-adrenoreceptor leads to elevation of NOX activity [Corbi et al., 2013]. It remains unclear whether NOXs have any effects on ROS-induced calpain-2 activation in cardiomyocytes of tail-suspended rats.

On the other hand, Ca^{2+}/calmodulin-dependent protein kinaseII (CaMK II), a critical transducer of Ca^{2+} signaling, is a multifunctional protein kinase which can phosphorylate a wide range of substrates and regulate numerous cellular functions. The δ isoforms of CaMK II predominate in the heart and two splice variants of CaMK II δ, δ_B, and δ_C, have been demonstrated to be present in the adult mammalian myocardium [Zhang and Brown, 2004]. CaMK II δ_B subunit localizes to the nucleus while CaMK II δ_C subunit localizes to the cytoplasm [Ramirez et al., 1997]. Emerging evidence suggests that overexpression of CaMK II δ_B induces cardiac hypertrophy [Zhang et al., 2002]; however, the functional role of CaMK II δ_B in the heart remains largely unknown. Overexpression of CaMK II δ_B in primary cardiac cells prevents the loss of the antiapoptotic protein Bcl-2, while specific silencing of CaMK II δ_B by small interfering RNA resists the formation of organized sarcomeres and decreases the expression of Bcl-2 [Little et al., 2009]. Recent study suggested that CaMK II might be a target for calpain in skeletal muscle [Otani et al., 2006]. Nuclear calpain regulates Ca^{2+}-dependent signaling via proteolysis of nuclear CaMK IV in cultured neurons [Tremper-Wells and Vallano, 2005]. Yet we understand little about relevant substrates and functional consequences of nuclear calpain in cardiomyocytes.

Herein, the aims of the present study were to observe the relationship between ROS level and nuclear calpain-2 activity in cardiomyocytes after ISO stimulation in tail-suspended rats. Furthermore, we would provide direct evidence that the activated calpain-2 in nucleus cleaved its substrate CaMK II δ_B and induced downregulation of Bcl-2. The mechanisms that increased susceptibility to apoptosis of cardiomyocytes through nuclear translocation of calpain-2 were elucidated in tail-suspended rats.

METHODS

CHEMICALS AND MATERIALS

The antibodies against cytochrome c, cytochrome oxidase IV (COX-IV), α-Tubulin, Bcl-2, and calpain-2 large subunit were purchased from Cell Signaling (Danvers, MA). The antibodies against cleaved caspase-3, cleaved caspase-9, lamin B1, CaMK II δ, Bax, and GAPDH were purchased from Santa Cruz (Santa Cruz, CA). The antibody against β-actin was purchased from Sigma–Aldrich (St. Louis, MO). NOX2 and NOX4 antibodies were purchased from Abcam (Cambridge, UK). Unless otherwise indicated, all chemicals were purchased from Sigma–Aldrich.

TAIL-SUSPENDED RAT MODEL

Tail-suspension of rat was performed as previously described by our laboratory [Chang et al., 2011] using a Morey–Holton method for 4 weeks [Morey-Holton and Globus, 2002]. 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 one day. Isoproterenol (ISO) was dissolved in physiological saline with equal amounts of vitamin C. A half of rats in the control or tail-suspended groups were intraperitoneally injected once with ISO (5 mg/kg body weight) at the day before the end of the experiment, whereas the other half in each experimental group with physiological saline. The rats in the recovery group were intraperitoneally injected once with physiological saline before 30 min of releasing suspension. The hearts in in vivo ISO treatment were used for RT-PCR, Western blot, TUNEL and zymography assay. All 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. Care was taken to

TABLE I. Comparison of noradrenaline (NA) content in plasma and left ventricular tissue in CON, SUS, and SUS + R groups

	CON	SUS	SUS + R
NA (ng/L)	106.15 ± 13.03	126.70 ± 16.63	328.36 ± 38.48[a]
NA (ng/g tissue)	421.17 ± 56.87	450.65 ± 68.33	591.45 ± 68.75[a]

Values are mean ± SEM, n = 8 rats in each group.
[a]$P < 0.05$ versus CON group.

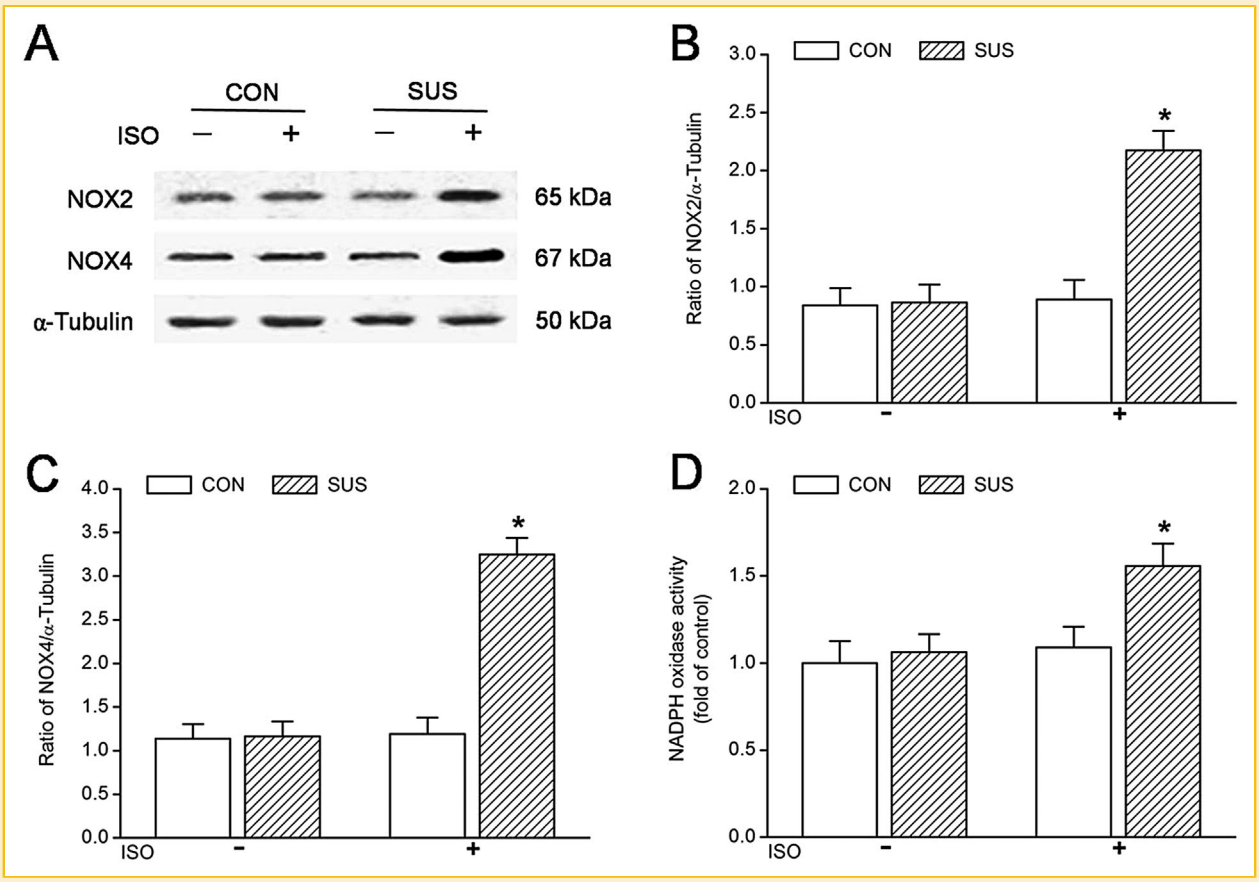

Fig. 1. Effect of ISO on NADPH oxidase (NOX) expression and activity in left ventricles of 4-week tail-suspended rats. A: Representative image of NOX2 and NOX4 protein expression by Western blot. B: Ratios of NOX2 to α-tubulin. C: Ratios of NOX4 to α-tubulin. D: NOXs activity. Data are expressed as mean ± SEM and represent four independent experiments. *$P < 0.05$ versus CON with ISO treatment.

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.

QUANTITATIVE REAL-TIME PCR

Total RNA was routinely extracted from left ventricular myocardium by TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) after ISO treatment. RNA (1 μg) was then reverse transcribed using the PrimeScrip RT reagent kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, China). Quantitative real-time PCR was performed using the SYBR Premix Ex Taq II kit (TaKaRa Biotechnology, Dalian, China). The relative expression values were normalized to the expression value of GAPDH. PCR primers were: Bcl-2, forward-AGTTCGGTGGGGTCATGTGTG, reverse-CCAGGTATGCACCCA-GAGTG; GAPDH, forward-TTCACCACCATGGAGAAGGC, reverse-GGCATGGACTGTGGTCATGA [Xiao et al., 2002]. Each sample was run in duplicate. The data were analyzed using the analysis module for absolute quantification in the Light Cycler Software 4.1. The relative expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001].

BIOCHEMISTRY ANALYSIS

Noradrenaline analysis. The concentration of noradrenaline in plasma and left ventricular myocardium of rats was analyzed with the Norepinephrine ELISA Kit (Abnova, Taipei, China) according to the manufacturer's instructions. The optical density was recorded using a micro-plate reader (μQuant; Bio-Tek Instruments, VT) at 450 nm. Means of triplicate results were compared among groups.

NADPH oxidase activity. NOX activity was determined by measuring the NADPH-dependent O_2^- production through superoxide dismutase-inhibitable cytochrome c reduction assay as described previously [Li et al., 2012]. Briefly, left ventricular myocardium of the rat was homogenized in lysis buffer after ISO treatment. Total protein was diluted (final concentration 1 mg/ml) and distributed in 96-well culture plates (final volume 200 μl/well). Cytochrome c (500 μM) and NADPH (100 μM) were added in the presence or absence of superoxide dismutase (200 U/ml) and incubated for 30 min at room temperature. Cytochrome c reduction was determined at 550 nm by a microplate reader. The results were expressed as a percentage of the control.

Zymography. Calpain activity was measured by casein zymography as previously described [Chang et al., 2011]. Briefly, 0.2% alkali-denatured casein 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-2 (50, 100, 200, 400, 800, and 1,000 ng; Calbiochem, Darmstadt, Germany) 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. The protein concentration was determined by a Bradford method, using bovine albumin fraction V as a standard.

IMAGE ACQUISITION AND QUANTIFICATION

Immunofluorescent cytochemistry. Adult rat cardiomyocytes were isolated and cultured as previously described [Chang et al., 2011].

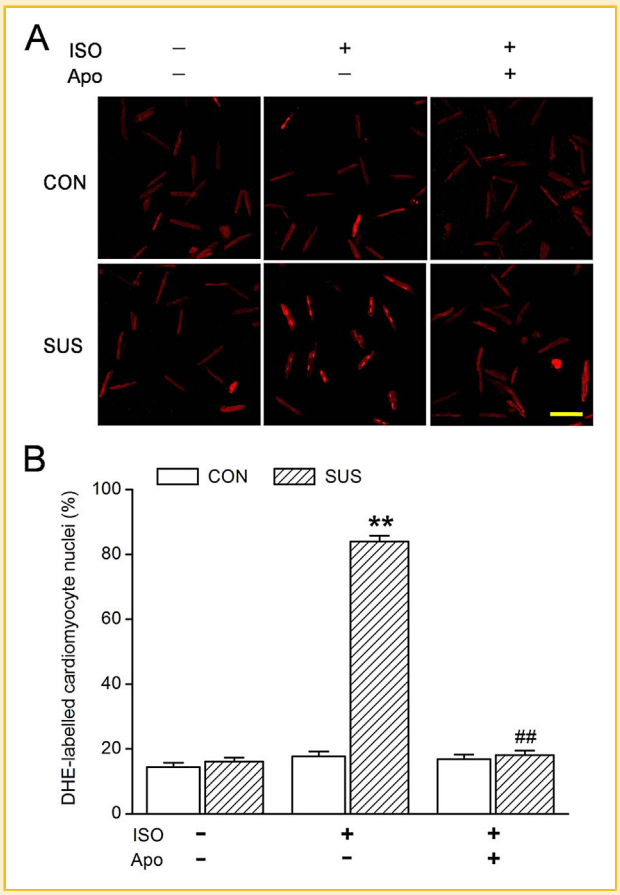

Fig. 2. Oxidative stress in cardiomyocytes in left ventricles of 4-week tail-suspended rats. A: Confocal fluorescence microscopy images of isolated cardiomyocytes from CON and SUS rats probed with DHE dye for assessment of the superoxide anion (O_2^-) formation. Scale bar = 150 μm. B: Quantitation of the DHE-labeled nuclei in cardiomyocytes. Apo, apocynin, an inhibitor of NADPH oxidase. Values are mean ± SEM; n = at least 200 cardiomyocytes from three rat hearts in each group. $^{**}P < 0.01$ versus CON with ISO treatment. $^{\#\#}P < 0.01$ versus SUS with ISO treatment.

Cardiomyocytes were cultured with or without 10 nM ISO for 24 h, or pre-incubated with 10 μM PD150606 or 200 μM apocynin or 2.5 mM N-acetyl cysteine (NAC) for 30 min, and subsequently treated with 10 nM ISO in combination with 10 μM PD150606 or 200 μM apocynin or 2.5 mM NAC for 24 h. After fixing in 4% paraformaldehyde for 30 min, cells were permeabilized in 0.1% Triton X-100/PBS for 30 min, blocked with 1% bovine serum albumin (BSA) in PBS for 60 min at room temperature, and then incubated with anti-calpain-2 (1:100; CST) at 4°C overnight. The slides were rinsed twice in PBS and incubated with TRITC-labeled goat anti-rabbit IgG for 60 min. Image was observed using a laser-scanning confocal microscope equipped with the FV10-ASW system (Olympus FV1000, Olympus Co., Ltd, Tokyo, Japan). Optical densitometry analysis of nuclear calpain-2 was performed using Olympus Fluoview image analysis software.

Superoxide production. Superoxide production was measured using dihydroethidium (DHE, Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) staining [Lai et al., 2011]. DHE is freely permeable to cells. Cytosolic DHE exhibits blue fluorescence but, when reacting with superoxide, will be oxidized to hydroxyethidium, intercalate within DNA, and stain the cell nucleus a bright fluorescent red. Cardiomyocytes attached to a laminin-coated glass slide were loaded with 10 μM DHE at 37°C for 30 min in a humidified black chamber. Fluorescent images were obtained using an Olympus FV1000 laser scanning confocal microscope with a 585 nm long-pass filter and analyzed with Olympus Fluoview image analysis software. The number of cardiomyocyte nuclei labeled by DHE was counted in each field and reported as percentage of the total number of myocyte nuclei evaluated by quantitative morphometric method.

Mitochondrial membrane potential (Δψm) assays. JC-1 is a kind of potentiometric dye, which exhibits membrane potential dependent-loss as JC-1 aggregates (polarized mitochondria) transition to JC-1 monomers (depolarized mitochondria) as indicated by the fluorescence emission shift from red to green. Briefly, myocytes were loaded with JC-1 in a final concentration of 5 μg/ml at 37°C for 20 min. The loaded-myocytes were rinsed and then scanned under a laser confocal microscope (Olympus FV1000). The Δψm was monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers (excited at 490 nm and measured at 530 nm) or JC-1 aggregates (excited at 525 nm and measured at 590 nm). Mitochondrial depolarization was indicated by an increase in the ratio of green to red fluorescence intensity.

TUNEL assay. TUNEL staining was strictly carried out according to the manufacturer's instructions (FragEL™ DNA Fragmentation Detection Kit, Calbiochem) to identify the apoptotic cells in paraffin sections as previously described [Chang et al., 2011]. Tissue sections were also counterstained with DAPI (0.5 μg/ml) and a membrane marker Texas red-conjugated wheat germ agglutinin (WGA 5 μg/ml, Molecular Probes) to identify the nucleus from the cardiomyocyte [Ang et al., 2010]. For each specimen, cells with positive nuclei staining from ten random fields were counted at a 60× water objective using a laser-scanning confocal microscope (Olympus FV1000). Quantitative analysis was presented as percentage of TUNEL-positive cardiomyocytes nuclei to total nuclei in each experimental group.

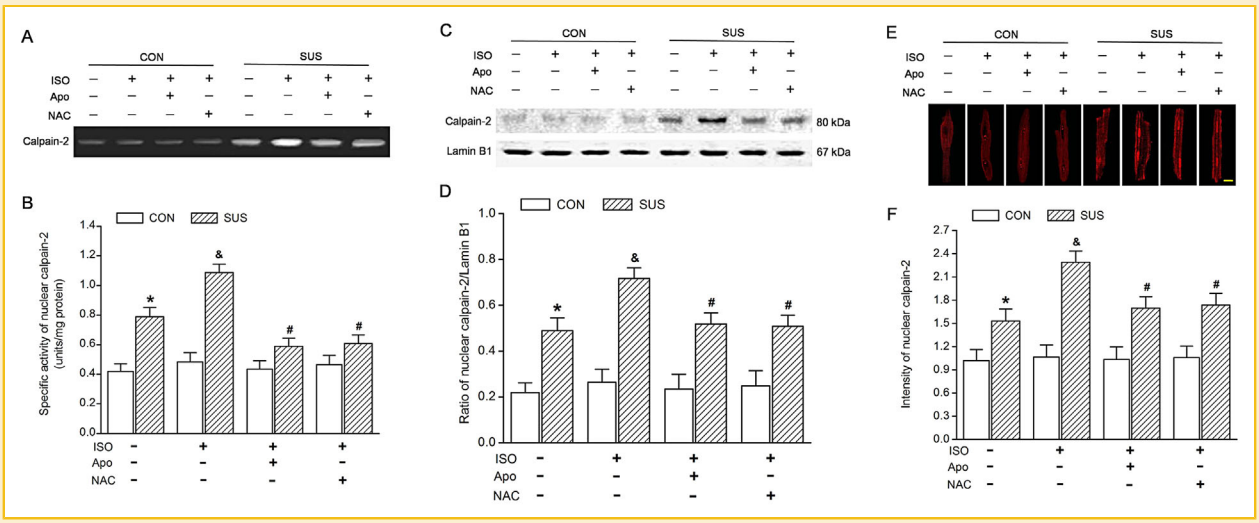

Fig. 3. Activity and expression of nuclear calpain-2 in left ventricle of 4 week tail-suspended rats. A: Representative casein zymography. B: Activity of calpain-2 in the isolated cardiomyocyte nuclei. C: Representative Western blots of calpain-2 in the isolated nuclei of cardiomyocytes. D: Ratio of calpain-2 to lamin B1 in cardiomyocyte nuclei. Values are mean ± SEM; n = 6 hearts in each group. E: Representative immunofluorescence images of calpain-2 distribution in cardiomyocytes. Scale bar = 20 μm. F: The fluorescence intensity of calpain-2 in nuclei was analyzed in three independent experiments. Values are mean ± SEM. At least 200 cardiomyocytes were analyzed in each group. $^{*}P < 0.05$ versus CON without ISO treatment. $^{\&}P < 0.05$ versus CON with ISO treatment. $^{\#}P < 0.05$ versus SUS with ISO treatment.

PROTEIN EXTRACTION AND WESTERN BLOT

Cytosolic and nuclear protein preparation was achieved with a Nuclear/Cytosolic Fractionation Kit (BioVision, Inc., Palo Alto, CA) following the instructions of the manufacturer. Mitochondrial and cytoplasmic fractions were prepared using the Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA) [Valente et al., 2013]. Purity of extracts was analyzed by Western blots using cytoplasmic (α-Tubulin), nuclear (lamin B1) and mitochondrial (COX-IV) markers. Western blot evaluations were undertaken as previously described [Chang et al., 2011]. Briefly, 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% BSA in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and incubated with primary NOX2 (1:1,000), NOX4 (1:1,000), calpain-2 large subunit (1:1,000), cytochrome c (1:1,000), COX-IV(1:1,000), α-Tubulin (1:1,000), Bcl-2 (1:1,000), Bax (1:1,000), lamin B1 (1:1,000), CaMK II δ (1:500), GAPDH (1:1,000), β-actin (1:10,000), cleaved caspase-3 (1:1,000) or cleaved caspase-9 (1:1,000) antibodies in TBS containing 0.1% BSA at 4°C overnight. Then the membranes were incubated with IRDye 680CW goat-anti mouse or IRDye800CW 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.

COIMMUNOPRECIPITATION

Colocalization of calpain-2 and CaMK II δ_B in cardiomyocyte nuclei was determined by coimmunoprecipitation. Nuclear fraction was prepared using a Nuclear/Cytosol Fractionation Kit (BioVision). Coimmunoprecipitation was performed according to a protocol from Pierce Classic IP Kit (26146; Thermo Fisher Scientific Inc., Rockford, IL). Five micrograms of calpain-2 antibody was combined with the pre-cleared cell nuclear extract lysate. All steps were performed at 4°C. Subsequently, the immune complex was captured and subjected to electrophoresis on SDS–PAGE. Then the blots were immunoblotted with CaMK II δ antibody and analyzed by Western blot as described above.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. 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 TREATMENT SIGNIFICANTLY INCREASES ROS PRODUCTION OF CARDIOMYOCYTES AS WELL AS EXPRESSION AND ACTIVITY OF NOXS IN THE MYOCARDIUM OF TAIL-SUSPENDED RATS

There was no difference in noradrenaline (NA) concentration of plasma and left ventricular myocardium between the CON and SUS groups. But NA concentration of plasma and left ventricular myocardium was significantly increased in the SUS + R group (Table I; $P < 0.05$). In order to simulate the changes in recovery heart after 4 weeks of tail-suspension, ISO was used as an agonist of β-adrenoreceptor in the present study.

The protein expression of NOX2 (Fig. 1A and B) and NOX4 (Fig. 1A and C) was significantly upregulated in ISO-treated cardiomyocytes

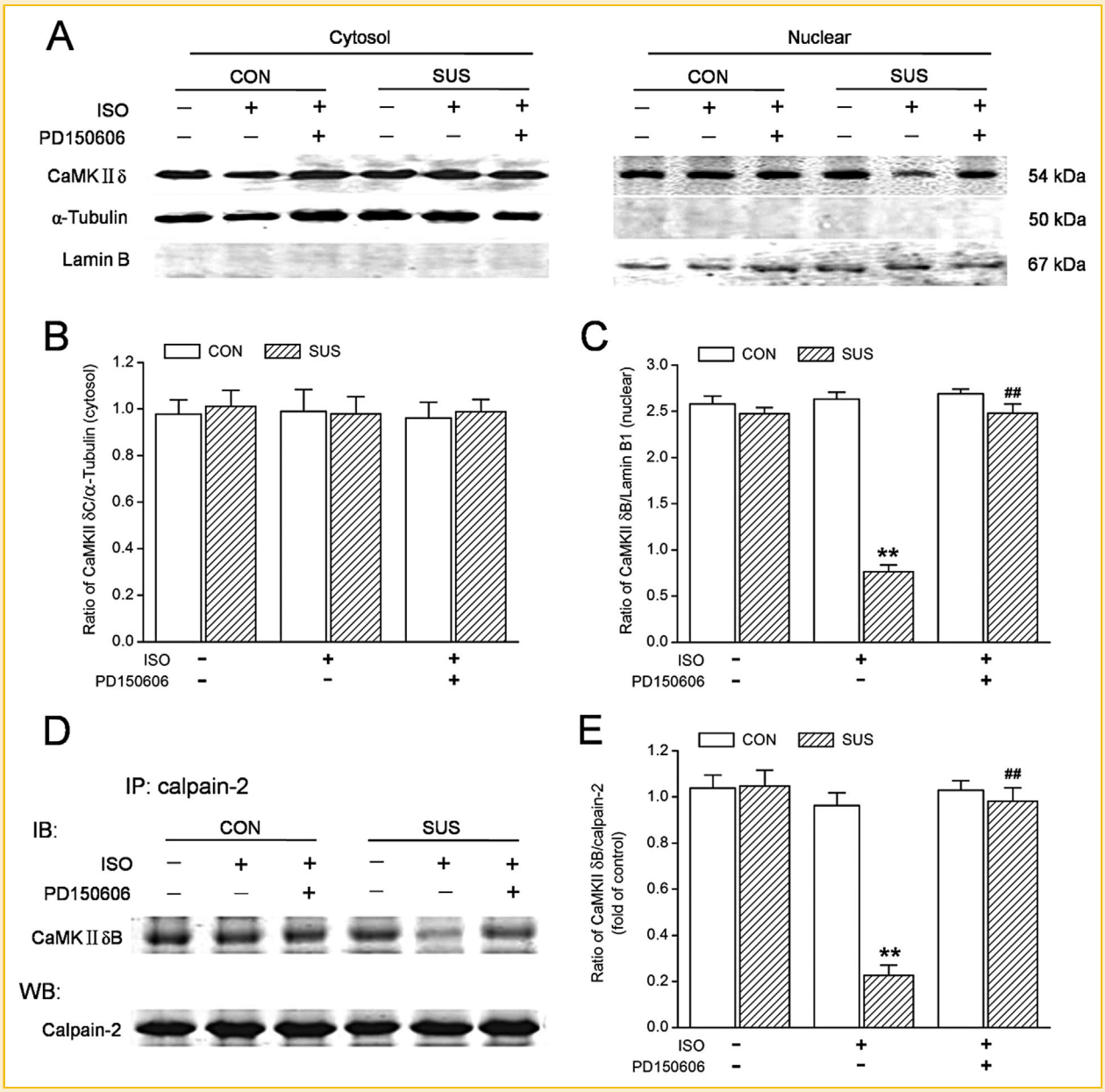

Fig. 4. Expression of CaMK II δ and the interaction between nuclear calpain-2 and CaMK II δ_B in left ventricles of 4-week tail-suspended rats. A: Typical Western blots of CaMK II δ protein. CaMK II δ_B and CaMK II δ_C localize to the nucleus and the cytoplasm, respectively. B, C: Statistic data of CaMK II δ_B and CaMK II δ_C protein abundance in the nuclei and the cytoplasm from left ventricle of rat hearts. D: Coimmunoprecipitation of calpain-2 and CaMK II δ_B in nuclear fractions of left ventricle in rat hearts. IB indicates immunoblots; IP, immunoprecipitation; WB, Western blots. E: Ratios of CaMK II δ_B to calpain-2. Values are mean ± SEM from three independent experiments. $^{**}P < 0.01$ as compared with CON group with ISO treatment. $^{\#\#}P < 0.01$ versus SUS with ISO treatment.

of SUS rats as compared with the control group. Consistent with these results, ISO treatment also significantly increased NOXs activity in cardiomyocytes of SUS rats (Fig.1D; $P < 0.05$).

As illustrated by representative images (Fig.2A) and quantitative analysis (Fig.2B), ISO treatment increased the DHE-positive nuclei of cardiomyocytes from 17% ± 1% in the CON to 83% ± 1% in the SUS group ($P < 0.01$). Apocynin, an inhibitor of NADPH oxidase, treatment abrogated ISO-induced ROS production in cardiomyocytes of SUS rats (Fig.2; $P < 0.01$). Thus, the upregulation of NOX activity was associated with increased ROS production in cardiomyocytes of SUS rats after ISO stimulation.

NADPH OXIDASE-DERIVED ROS INDUCES ACTIVATION AND NUCLEAR TRANSLOCATION OF CALPAIN-2

Both calpain-2 activity (Fig.3A and B) and expression (Fig.3C and D) showed a significant increase in isolated nuclei of cardiomyocytes in the SUS rats ($P < 0.05$). The activity and expression of calpain-2 were further increased under ISO treatment in the isolated nuclei of

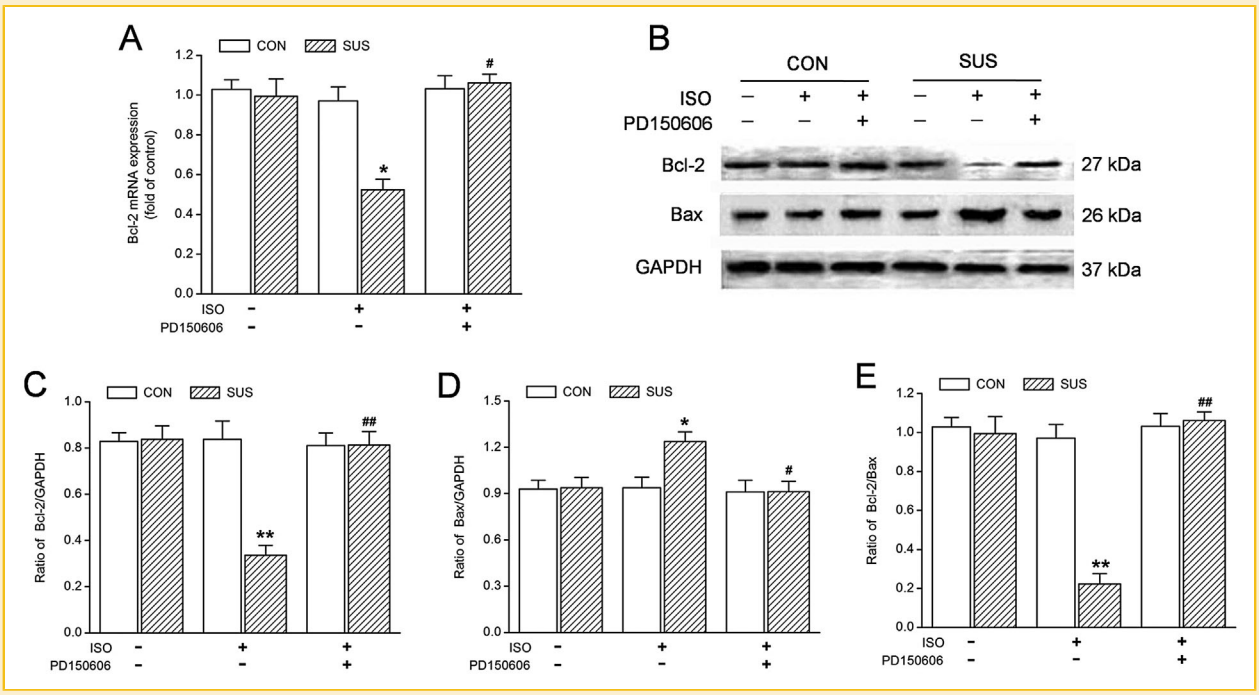

Fig. 5. Expression of Bcl-2 mRNA and expression of Bcl-2 and Bax proteins in rat left ventricles. A: The statistic data of Bcl-2 mRNA expression assayed by quantitative real-time PCR. B: Representative Western bolts of Bcl-2, Bax, and GAPDH in rat left ventricles. C: The ratio of Bcl-2 to GAPDH. D: The ratio of Bax to GAPDH. E: The ratio of Bcl-2 to Bax. Values are mean ± SEM from three independent experiments. $^{*}P < 0.05$ or $^{**}P < 0.01$ as compared with CON group with ISO treatment. $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ versus SUS with ISO treatment.

cardiomyocytes from SUS rats ($P < 0.01$). Inhibition of NOXs with apocynin diminished ISO-induced calpain-2 activity and downregulated calpain-2 expression in isolated nuclei of cardiomyocytes ($P < 0.05$). Similarly, scavenging ROS by NAC blocked calpain-2 activation and decreased calpain-2 expression in nuclei of ISO-stimulated cardiomyocytes ($P < 0.05$).

To further confirm the effects of NOXs-produced ROS on nuclear translocation of calpain-2, the isolated cardiomyocytes were observed by an immunofluorescent cytochemical technique. Confocal microscope immunofluorescence images indicated that the nuclear accumulation of calpain-2 in cardiomyocytes was greater in the SUS group than that in the CON group (Fig.3E). ISO treatment promoted the nuclear translocation of calpain-2 in SUS rat cardiomyocytes ($P < 0.01$), while inhibiting NOXs with apocynin or scavenging ROS by NAC reduced ISO-induced nuclear translocation of calpain-2 (Fig.3F; $P < 0.05$).

ISO INCREASES THE CONSTITUTIVE BINDING BETWEEN CALPAIN-2 AND CAMK II Δ_B, THEN PROMOTES THE DEGRADATION OF CAMK II Δ_B IN CARDIOMYOCYTE NUCLEI OF TAIL-SUSPENDED RATS

The protein level of CaMK II δ_C in the cytosol of cardiomyocytes was unaltered between the CON and SUS group with or without ISO treatment (Fig.4A and B). In contrast, the protein level of CaMK II δ_B in the nuclei of cardiomyocytes was profoundly reduced after ISO treatment in the SUS group, whereas using a calpain-specific inhibitor, PD150606, blocked the reduction of CaMK II δ_B (Fig.4A and C; $P < 0.01$).

The coimmunoprecipitation showed that calpain-2 was constitutively bound to CaMK II δ_B in nuclei of cardiomyocytes, but this binding was reduced after ISO stimulation in the SUS group (Fig.4D and E; $P < 0.01$). Inhibition of calpain by PD150606 restored the binding between calpain-2 and CaMK II δ_B in cardiomyocyte nuclei of the SUS group ($P < 0.01$).

INHIBITION OF CALPAIN-2 RESISTS ACTIVATION OF MITOCHONDRIAL APOPTOTIC PATHWAY IN CARDIOMYOCYTES OF TAIL-SUSPENDED RATS

The mRNA and protein expressions of Bcl-2 were significantly decreased (Fig.5A, B and C; $P < 0.05$ or $P < 0.01$), but Bax protein expression was markedly increased in the ISO-treated cardiomyocytes of SUS group (Fig.5D; $P < 0.05$). Thus, the ratio of Bcl-2 to Bax protein was decreased in the ISO-treated cardiomyocytes of SUS rats. PD150606 resisted the downregulation of Bcl-2 mRNA and protein, increase in Bax protein, and reduction in ratio of Bcl-2 to Bax after ISO stimulation in tail-suspended rat hearts (Fig.5E; $P < 0.01$).

The ratio of green monomer-to-red aggregate JC-1 (G-R ratio) is used as an indicator of mitochondrial membrane potentials ($\Delta\psi m$) of cardiomyocytes. The G-R ratio is inversely proportional to the depolarization of $\Delta\psi m$. The G-R ratio was increased after ISO treatment in cardiomyocytes of SUS rats, however, calpain inhibitor PD150606 reduced G-R ratio to normal level during ISO treatment in cardiomyocytes of SUS rats (Fig.6A and B; $P < 0.01$).

As shown in Fig. 6C, Fig. 6D and Fig. 6E, ISO increased cytosolic cytochrome c level and decreased mitochondrial cytochrome c level

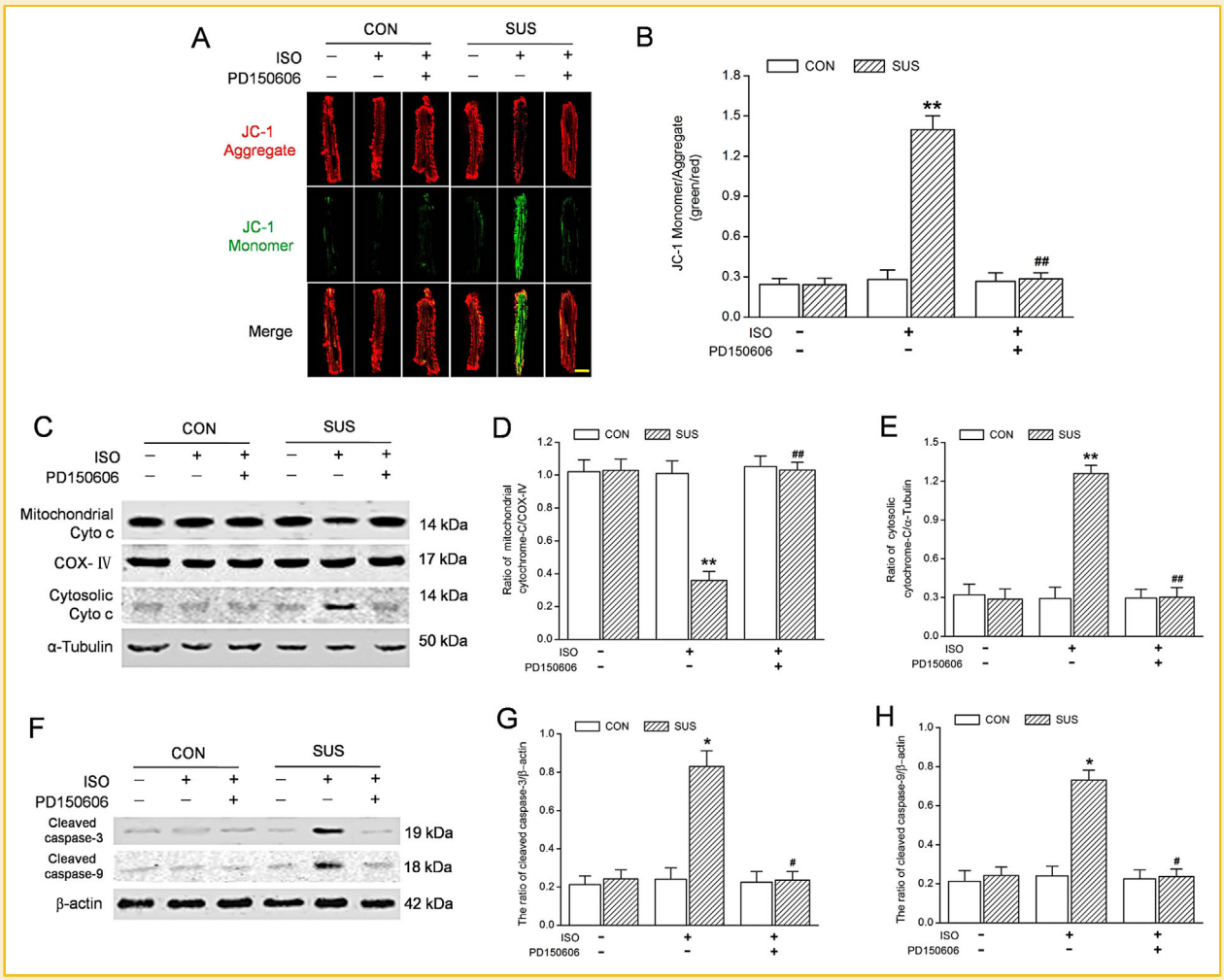

Fig. 6. ISO activated mitochondrial apoptotic pathway via calpains in tail-suspended rats. A: Representative fluorescent images of cardiomyocytes stained with JC-1 probe in cardiomyocytes. Scale bar = 20 μm. B: Fluorometric quantification of JC-1 staining. Green monomer-to-red aggregate ratio indicates depolarization of $\Delta\psi m$. An increase in the bar indicated a shift in the fluorescence ratio correlating with an increase in mitochondrial depolarization. Values are mean ± SEM with the number of 8 cardiomyocytes recorded from three independent experiments. C: Western blot of cytochrome c distribution was done on mitochondrial and cytosolic fractions from rat left ventricle. Cytochrome oxidase IV (COX-IV) was used as a marker of mitochondria. D, E: Ratios of cytochrome c to COX-IV in mitochondrial fractions and to α-tubulin in cytosolic fractions. Values are mean ± SEM from three independent experiments. F: Representative Western bolts of cleaved caspase-3 and cleaved caspase-9 (activated) in rat left ventricles. G, H: The changes in caspase-3 and caspase-9 activities. Values are mean ± SEM from three independent experiments. $^{*}P < 0.05$ or $^{**}P < 0.01$ as compared with CON group with ISO treatment. $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ versus SUS with ISO treatment.

in cardiomyocytes of SUS rats ($P < 0.01$). PD150606 inhibited ISO-induced mitochondrial cytochrome c release into the cytoplasm in cardiomyocytes of SUS rats ($P < 0.01$).

Caspase-3 and caspase-9 are activated by ISO stimulation in cardiomyocytes of SUS rats, as demonstrated in Fig. 6F by Western blotting. Densitometric analysis (Fig.6G and H) showed that cleaved caspase-3 and cleaved caspase-9 were increased by ISO treatment in tail-suspended rat hearts. Inhibition of calpain-2 with PD150606 reversed this response ($P < 0.05$).

INHIBITION OF NOXS OR CALPAIN-2 PREVENTS ISO-INDUCED CARDIOMYOCYTE APOPTOSIS IN TAIL-SUSPENDED RATS

TUNEL assay showed that ISO treatment markedly increased the percentage of apoptotic cardiomyocytes in the SUS group (Fig.7A and B; $P < 0.01$). The proapoptotic effects of ISO were attenuated by treatment of apocynin or PD150606 (Fig.7A and B; $P < 0.05$).

DISCUSSION

In the present study, the data showed for the first time that ISO-enhanced susceptibility to apoptosis in cardiomyocytes was mediated by increased NOXs-derived ROS which activated nuclear translocation of calpain-2, subsequently nuclear calpain-2 degraded CaMK II δ_B which reduced the ratio of Bcl-2 to Bax, and finally the mitochondrial apoptotic pathway was triggered in tail-suspended rat cardiomyocytes.

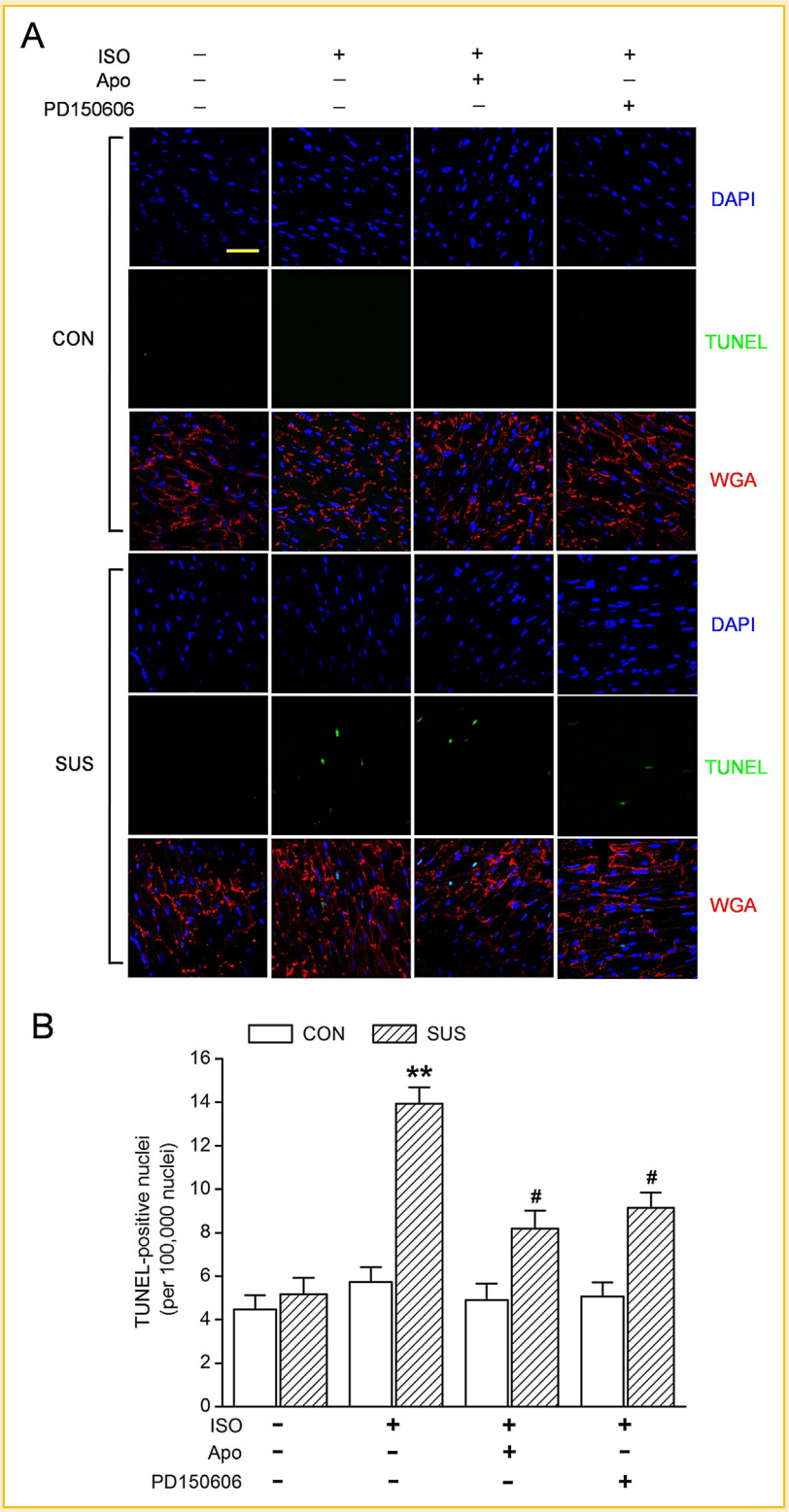

Fig. 7. Role of NADPH oxidase and calpain-2 on cardiomyocyte apoptosis in left ventricles of 4-week tail-suspended rats. A: TUNEL assay. Green is TUNEL-positive cell nuclei. Blue represents cell nuclei counter stained with DAPI. Red represents wheat-germ agglutinin (WGA) stain of myocardial interstitial tissue. Scale bar = 50 μm. B: 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 ± SEM. $^{**}P < 0.01$ versus CON with ISO treatment. $^{\#}P < 0.05$ versus SUS with ISO treatment.

ISO INCREASES NOXS ACTIVITY, AND THEN NOXS-DERIVED ROS PRODUCTIONS PROMOTE NUCLEAR TRANSLOCATION OF CALPAIN-2 IN CARDIOMYOCYTES

This study showed that NA content in plasma and left ventricular tissue increased in the recovery rats from 4 week tail-suspension (SUS + R group), but not in the tail-suspended rats. Selective increase in β_1- and β_2-adrenoreceptor responsiveness is a characteristic change in the heart after simulated microgravity [Convertino et al., 1997]. NA has been shown to stimulate apoptosis through activation of β-adrenoreceptor [Communal et al., 1998], and also induce calpain-1 activation in adult rat cardiomyocytes [Li et al., 2009a]. The increased NA release occurred when the tail-suspension of rat was removed, which was in accordance with the previous study that 1-day recovery increased cardiomyocyte apoptosis which was blocked by propranolol, a blocker of β-adrenergic receptor [Chang et al., 2011]. The enhanced activation of β-adrenoreceptor increased susceptibility of cardiomyocyte apoptosis in tail-suspended rats [Chang et al., 2011]. Therefore, in the present study, the β-adrenergic agonist ISO was used as a proapoptotic factor to elucidate the underlying mechanisms of cardiomyocyte apoptosis.

In the cardiomyocyte, ROS can be formed by several mechanisms, including mitochondrial electron transport chain, nitric oxide synthase, NOXs, xanthine oxidase, cytochrome P450, and lipoxygenase(LOX)/cyclooxygenase (COX) pathways [Kevin et al., 2005]. Among these origins, NOXs are a major source of ROS in the myocardium [Kumar and Jugdutt, 2003]. There are two NOX isoforms, NOX2 and NOX4, in cardiomyocytes [Nabeebaccus et al., 2011]. NOX-mediated ROS production is implicated in α_1-adrenoreceptor-stimulated hypertrophy of adult rat ventricular myocytes [Xiao et al., 2002]. Transgenic activation of β_2-adrenoreceptor leads to elevation of NOX activity [Corbi et al., 2013]. In the present study, β-adrenoreceptor agonist ISO not only increased expression of NOXs, but also elevated NOX activity in the myocardium of tail-suspended rats (Fig.1). Thus, ISO induced an increase in ROS productions in cardiomyocytes of tail-suspended rats (Fig.2). ISO increases the peak of intracellular Ca^{2+} transient, and endogenously produced ROS further elevate the peak of intracellular Ca^{2+} transient in cardiomyocytes [Andersson et al., 2011]. In endothelial cells, it has been suggested that ROS mediates the elevation in intracellular Ca^{2+} and subsequent calpain activation, while inhibition of NOX activity with apocynin or DPI attenuates the increase in intracellular Ca^{2+} [Dong et al., 2009]. Besides, calpain-2 activation can be prevented by the ROS scavenger in retinal photoreceptor cells [Sanvicens et al., 2004; Su et al., 2010]. The underlying mechanisms of ROS activating calpain-2 are unclear until now; ROS may increase intracellular Ca^{2+} or p38/JNK activation which activates calpain-2 in retinal photoreceptor cells or HEK-293 cells [Sanvicens et al., 2004; Su et al., 2010]. In the present study, NOXs inhibition or scavenging ROS could prevent nuclear translocation and activation of calpain-2 in ISO-stimulated cardiomyocytes of tail-suspended rat (Fig.3). Thus, ROS production derived by NOXs is an important factor for calpain-2 activation. This is consistent with a previous report demonstrating that activation of β-adrenoreceptor induces NOX activation, which contributes to apoptosis [Remondino et al., 2003].

NUCLEAR CALPAIN-2 MAY DEGRADE CAMK II Δ_B WHICH MODULATES BCL-2 EXPRESSION AND FURTHER FACILITATES CARDIOMYOCYTE APOPTOSIS THROUGH MITOCHONDRIAL APOPTOTIC PATHWAY

Cardiac CaMK II δ_B and CaMK II δ_C, localized in nuclear and cytosolic compartments, respectively, were inversely regulated in response to oxidative stress, and that in contrast to CaMK II δ_C, CaMK II δ_B served as a potent suppressor of cardiomyocyte apoptosis [Peng et al., 2010]. CaM-binding proteins are particularly vulnerable to cleavage by calpain [Wang et al., 1989]. CaMK IV was cleaved by calpain in SH-SY5Y human neuroblastoma cells undergoing apoptosis [McGinnis et al., 1998]. In the present study, we identified CaMK II δ_B as a direct target of calpain-2 protease in rat cardiomyocytes nuclei (Fig. 4D and E). By using coimmunoprecipitation technique, we found that the large subunit of calpain-2 was constitutively bound to CaMK II δ_B in cardiomyocyte nuclei, and that calpain-2 activation by ISO exposure increased this binding. Calpain-2 is heterodimer containing a 28-kDa small regulatory subunit and an 80-kDa large catalytic subunit [Goll et al., 2003]. The large subunit of calpain has its catalytic domain near the N-terminal region and has a calmodulin-like E-F hand Ca^{2+}-binding domain at the C-terminal region [Ohno et al., 1984]. Besides, CaMK II δ_B contains a calmodulin-binding domain. It is conceivable that the calmodulin-like domain of calpain can bind to the calmodulin-binding domain of the substrate proteins. Once binding is achieved, the calpain catalytic domain proceeds to cleave its bound substrate [Wang et al., 1989].

Bcl-2 family proteins are major regulators of the mitochondrial apoptotic pathway and play an important role in regulating cardiomyocyte apoptosis [Gustafsson and Gottlieb, 2007]. Overexpression of CaMK II δ_B in primary cardiac cells prevents the loss of the antiapoptotic protein Bcl-2, in contrast specific silencing of CaMK II δ_B by small interfering RNA decreases the expression of Bcl-2 [Little et al., 2009]. Simultaneous inhibition of ROS and calpain is required for preventing Bax activation and cell death [Sobhan et al., 2013]. Consistent with these studies we had observed the decreased nuclear CaMK II δ_B expression as well as the decreased ratio of Bcl-2 to Bax in cardiomyocytes treated with ISO in tail-suspended rats (Fig.4 and Fig. 5). However, pretreatment of cardiomyocytes with calpain inhibitor completely prevented the degradation of CaMK II δ_B and decrease in Bcl-2/Bax ratio under ISO stimulation indicating that both nuclear calpain-2 and CaMK II δ_B cooperated for Bax activation and Bcl-2 suppression. These results showed that the proapoptotic mechanism of nuclear calpain-2 in cardiomyocytes induced by oxidative stress would be, at least in part, functionally attributed to its suppression of Bcl-2 and activation of Bax.

It is known that activation of mitochondrial apoptotic pathway is featured by the alteration of permeability of mitochondrial membrane and the release of cytochrome c into cytoplasm to activate the caspase cascade [Green and Kroemer, 2004]. Here, we showed that pharmacological inhibitor of calpains (PD150606) protected against the activation of mitochondria apoptotic pathway in cardiomyocytes under ISO treatment in tail-suspended rats (Fig.6). The mitochondrial membrane potentials ($\Delta\psi m$) was reduced, meanwhile, the protein level of released cytochrome c was markedly increased by ISO stimulation (Fig.6). In line with the TUNEL results (Fig.7), the expression of cleaved caspase-3 and cleaved caspase-9,

markers of apoptosis, were also found to be significantly increased in ISO-treated cardiomyocytes. Coincidently, simulated microgravity resulted in cerebrovascular mitochondrial dysfunction, and cross-talk between NOX and mitochondria participated in the process [Zhang et al., 2014]. Together, these data support an essential role of nuclear calpain-2 in the regulation of the mitochondrial machinery through modulating Bcl-2 levels.

In summary, the present results reveal a signaling transduction pathway that nuclear calpain-2 mediates apoptosis in cardiomyocytes of tail-suspended rats. In this cascade, ISO-induced ROS production appears as the initial trigger for the subsequent activation and nuclear translocation of calpain-2 and the onset of the apoptotic process by targeting CaMK II δ_B in cardiomyocytes nuclei. These findings not only define a novel cardiac apoptosis pathway mediated by calpain-2, but also imply that nuclear calpain-2 upregulation or activation might be a crucial pathogenic element and a potentially therapeutic target for oxidative stress-induced cardiomyocyte apoptosis.

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