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Original Paper

Role of the Calcium-Sensing Receptor in Cardiomyocyte Apoptosis via the Sarcoplasmic Reticulum and Mitochondrial Death Pathway in Cardiac Hypertrophy and Heart Failure

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Key Words
Calcium-sensing receptor • ER stress • Mitochondria • Apoptosis • Cardiac hypertrophy • Heart failure

Abstract
Aims: Alterations in calcium homeostasis in the intracellular endo/sarcoplasmic reticulum (ER/SR) and mitochondria of cardiomyocytes cause cell death via the SR and mitochondrial apoptotic pathway, contributing to ventricular dysfunction. However, the role of the calcium-sensing receptor (CaR) in cardiac hypertrophy and heart failure has not been studied. This study examined the possible involvement of CaR in the SR and mitochondrial apoptotic pathway in an experimental model of heart failure. ***Methods and Results:*** In Wistar rats, cardiac hypertrophy and heart failure were induced by subcutaneous injection of isoproterenol (Iso). Calindol, an activator of CaR, and calhex231, an inhibitor of CaR, were administered by caudal vein injection. Cardiac remodeling and left ventricular function were then analyzed in these rats. After 2, 4, 6 and 8 weeks after the administration of Iso, the rats developed cardiac hypertrophy and failure. The cardiac expression of ER chaperones and related apoptotic proteins was significantly increased in the failing hearts. Furthermore, the expression of ER chaperones and the apoptotic rate were also increased with the administration of calindol, whereas the expression of these proteins was reduced with the treatment of calhex231. We also induced cardiac hypertrophy and failure via thoracic aorta constriction (TAC) in mice. After 2 and 4 weeks of TAC, the expression of ER chaperones and apoptotic proteins were increased in the mouse hearts. Furthermore, Iso induced ER stress and apoptosis in cultured cardiomyocytes, while pretreatment with calhex231 prevented ER stress and protected the myocytes against apoptosis. To further investigate the effect of CaR on the concentration of intracellular calcium, the calcium concentration in the SR and mitochondria was determined with Fluo-5N and x-rhod-1 and the mitochondrial membrane potential was examined with JC-1 using laser con-

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focal microscopy. After treatment with Iso for 48 hours, activation of CaR reduced $[Ca^{2+}]_{SR}$, increased $[Ca^{2+}]_m$, decreased the mitochondrial membrane potential, increased the expression of ER stress chaperones and related apoptotic proteins, and induced the release of cytochrome c from the mitochondria. ***Conclusions:*** Our results demonstrated that CaR activation caused Ca^{2+} release from the SR into the mitochondria and induced cardiomyocyte apoptosis through the SR and mitochondrial apoptotic pathway in failing hearts.

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Introduction

Heart failure is a major cause of cardiovascular death in China [1]. Previous work has indicated that dysfunction of the intracellular Ca^{2+} cycle is crucial for the pathogenesis of heart failure [2]. The endoplasmic/sarcoplasmic reticulum (ER/SR) is an intracellular Ca^{2+} storage organelle, which is sensitive to alterations in Ca^{2+} homeostasis and disturbances in its environment. The depletion of Ca^{2+} from the SR lumen can disrupt SR function, resulting in ER stress. Prolonged and severe ER stress results in organelle damage and dysfunction, ultimately leading to apoptosis [3, 4]. It has been reported that ER stress is involved in heart diseases that contribute to heart failure, including hypertension, myocardial ischemia, and dilated cardiomyopathy [5]. The heart is the most mitochondria-rich organelle, as mitochondria occupy approximately 30% of the volume of a ventricular cardiomyocyte. Mitochondria are the organelles responsible for the production of ATP and are the gatekeepers of cellular viability. In heart failure, mitochondrial ATP production is decreased and its apoptotic pathway is activated [6, 7]. Many studies have confirmed the important role of biochemical cross-talk between the sarcoplasmic reticulum and mitochondria in normal cardiomyocyte survival [8-11]. Although the SR and mitochondria have different functions, their coordination is necessary to support synchronous cardiomyocyte contraction/relaxation as well as cell viability. SR and mitochondrial dysfunction are associated with heart failure [12]. However, how the interplay between the SR and mitochondria contributes to heart failure is unclear.

The calcium-sensing receptor (CaR) is G protein-coupled receptor that stimulates the phosphatidyl inositol-specific phospholipase C (PLC), leading to the generation of the secondary messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [13, 14]. The mobilization of Ca^{2+} from intracellular stores by IP_3 via the activation of a specific inositol 1,4,5-trisphosphate receptor (IP_3R) signaling pathway could release Ca^{2+} from the SR to increase the microdomain Ca^{2+} concentration ($[Ca^{2+}]$) at focal contacts, known as mitochondria-associated ER membranes (MAM) between the ER/SR and mitochondria, and then activate the mitochondrial calcium uniporter (MCU). Recent studies have suggested that IP_3Rs are highly compartmentalized in MAMs, providing direct mitochondrial Ca^{2+} signaling [8].

In a previous study, we demonstrated that CaR is involved in apoptosis in isolated adult rat hearts and in rat neonatal cardiomyocytes during hypoxia/reoxygenation (H/Re) [14]. Although CaR is known to elevate the concentration of intracellular calcium, the in-depth mechanism has not been delineated. In the present study, we tested the hypothesis that the SR and mitochondrial apoptotic pathway plays a role in cardiomyocyte apoptosis in heart failure through CaR activation.

Materials and Methods

Materials

Isoproterenol (Iso), calindol, calhex231, sodium tauroursodeoxy cholate were purchased from Sigma-Aldrich (St. Louis, MO,USA). Antibodies for Calcium sensing receptor (CaR), type $3IP_3R$, ATF6, GRP78, caspase-12, CHOP (GADD153), cytochrome c, VDAC, β-actin and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse-radish peroxidase-conjugated secondary antibodies (goat anti-rabbit immunoglobulin G and rabbit anti-mouse immunoglobulin G) and enhanced chemiluminescence reagents were from Pierce Biotechnology (now part of Thermo Fisher Scientific, Rockford, IL). Polyvinylidene difluoride

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(PVDF) membranes were from Whatman (now part of GE Healthcare Life Sciences, Buckinghamshire, UK). TUNEL kit was from Roche (Darmstadt, Germany). Fluo-5N and X-rhod-1 AM were from Molecular Probe (Molecular Probe, USA).

Animals

Male Wistar rats (200-250 g) were obtained from the Experimental Animal Center of Harbin Medical University (Harbin, People's Republic of China). All animal experimental protocols complied with the 'Guide for the Care and Use of Laboratory Animals' published by the United States National Institutes of Health. The study was approved by the Institutional Animal Research Committee of Harbin Medical University. All animals were housed at the animal care facility of Harbin Medical University at 25°C with 12/12-h light/dark cycles and allowed free access to normal rat chow and water throughout the study period. Rats were randomly assigned to different treatment groups.

Experimental groups

β-adrenergic stimulation can be induced by subcutaneous injection of high dose Iso as previously reported [15]. Eighty rats were randomly divided into four groups. In group 1 (n= 20), the rats were subcutaneously injected with saline for 4 days as a control. In group 2 (Iso, n= 20), the rats were subcutaneously injected with Iso (170 mg/kg/d in saline) for 4 days to induce cardiac hypertrophy and heart failure. In group 3 (Iso-A, n= 20), the rats were subcutaneously injected with calindol (10 μmol/kg/d in saline) for 2, 4, 6, or 8 weeks after administration of Iso for 4 days. Calindol is a specific activator of CaR. In group 4 (Iso-I, n= 20), the rats were subcutaneously injected with calhex231 (10 μmol/kg/d in saline) for 2, 4, 6, or 8 weeks after administration of Iso for 4 days. Calhex231 is a specific inhibitor of CaR.

Thoracic aorta constriction (TAC)

Pressure overload and cardiac hypertrophy were induced by thoracic aorta constriction (TAC) in male Kunming mice according to a previously described method [16]. Thirty-two rats were randomly assigned to four experimental groups. In group 1 (n =20), sham-operated animals served as controls. In group 2 (n= 20), cardiac hypertrophy was induced by TAC without further treatment. In group 3 (n =20), TAC was performed and the rats were treated with calindol (10 μmol/kg/d in saline) for 2 or 4 weeks after the operation. In group 4 (n= 20), TAC was performed, and the rats were treated with calhex231 (10 μmol/kg/d in saline) for 2 or 4 weeks after the operation.

Echocardiographic Assessment of Regional Myocardial Function

Rats were anesthetized with 2% pentobarbital sodium (50 mg kg^{-1}, ip). Echocardiographic images were obtained with a Vivid 7 Dimension echocardiographic machine (GE Healthcare, Waukesha, WI, USA). Regional wall thickening, myocardial contractile function and wall motion were determined by a single experienced investigator in a blinded fashion. These images and parameters were recorded for subsequent review and analysis.

HE staining Analysis

Formalin fixed hearts were embedded in paraffin, sectioned into 4 μm slices, and stained with hematoxylin and eosin (H&E). Cardiac myocyte crosssectional diameter was measured. The distance across the myocardial cell at its narrowest plane across the nucleus was measured in 75 cells from each LV. The mean diameter was calculated for the LV in each rat.

TUNEL (terminal deoxynucleotidyl transferasemediated dUTP nick-end labelling) assay

The TUNEL assay was used to detect the apoptosis of cardiomyocytes in rat hearts. The heart tissues (n=3 for sham group and n=5 for each HF group) were fixed in 4% paraformaldehyde overnight, dehydrated and then embedded in paraffin. The assay was tested by a TdT- DNA Fragmentation Detection kit acquired from Roche, and the empirical procedure was performed according to the kit's protocol.

Mitochondrial ultrastructure changes observed in the heart tissues by electron microscope

Mitochondrial ultrastructure changes of cardiomyocytes in our present study was detected by EM (electron microscope) [14]. Mitochondria isolated from heart tissue was fixed with 2.5% glutaldehyde and

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1.5% paraldehyde, washed in PBS, fixed in osmium tetroxide, dehydrated in an ethanol series, embedded in epoxy resin, and then examined with a Philips CM 120 electron microscope.

Isolation of neonatal rat ventricular cardiomyocytes (NVCMs)

Primary cultures of neonatal rat cardiomyocytes were performed as previously described [17]. Newborn Wistar rats (1-3 days) were used for this study. The rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the China National Institutes of Health. Briefly, hearts from male Wistar rats (1-3 days old) were minced and dissociated with 0.25% trypsin. Dispersed cells were seeded at 2×10^5 cells/cm^2 in 60-mm culture dishes with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and then cultured in a 5% CO_2 incubator at 37°C. At 72 h post-culturing with 10% FBS/DMEM, the cells were randomly divided into six groups: (1) control group: cells were continuously cultured for 48h with 10% FBS/DMEM; (2) Iso group: cells were placed in culture medium for 48 h with 5 μM Iso; (3) Iso+calindol(Iso-A): cells were placed in culture medium for 48 h with 5μM Iso and 10 μM calindol; (4) Iso+calhex231+calindol (Iso-I): cells were placed in culture medium for 48 h with 5 μM Iso and 10 μM calhex231 and 10 μM calindol; (5) Iso+2-APB+calindol (Iso-2-APB-A): cells were placed in culture medium for 48 h with 5 μM Iso and 20 μM 2-APB and 10 μM calindol. 2-APB is an inhibitor of IP_3Rs; (6): Iso+Rutheium red +calindol (Iso-Ru R-A): cells were placed in culture medium for 48 h with 5 μM Iso and 10 μM Rutheium red and 10 μM calindol. Rutheium red is a blocker of mitochondrial calcium unitransporter.

Western-blot analysis of proteins in left ventricular tissues and neonatal rat ventricular cardiomyocytes (NVCMs)

The protein concentration of each sample was quantified using the enhanced BCA (bicinchoninic acid) Protein Assay kit (Beyotime, Nantong, China). Cell lysates and heart extracts were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE) and blotted on PVDF membranes (Millipore). Membranes were incubated with antibodies directed against CaR, type 3 IP_3R, GRP78, ATF6, CHOP and caspase-12 protein. A secondary antibody was AP-IgG (Promega). The volume of the protein bands was quantified by a Bio-Rad Chemi Doc™ EQ densitometer and a Bio-Rad Qantity One software (Bio-Rad laboratories, Hercules, USA).

Separation of cytosolic and mitochondrial proteins

Isolation of mitochondria protein from left ventricular tissue was performed according to the manufacturer's protocol (Beyotime, Nantong, China) [18]. Protein concentrations were determined using bicinchoninic acid Protein Assay kit (Beyotime, Nantong, China).

Measurement of SR Ca^{2+} and mitochondrial Ca^{2+} concentrations with Fluo-5N and X-rhod-1 AM, respectively

To determine $[Ca^{2+}]_{SR}$, cardiomyocytes were loaded with Fluo-5N acetoxymethyl ester (10 μM) were performed as previously described [19]. For intact myocytes, the superfusate contained (in mM) 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.4, 23˚C). For permeabilization, myocytes were exposed to a solution containing (in mM) 0.1 EGTA, 10 HEPES, 120 K-aspartate, 1 free $MgCl_2$, 5 ATP, 10 reduced glutathione, 5 phosphocreatine [pH 7.4] and then permeabilized with saponin (50 μg / ml) for 20 sec. Excitation was set at 488 nm and emission was measured at 530 nm at room temperature.

$[Ca^{2+}]_m$ was determined according to the manufacturer's instructions (Molecular Probes). In brief, the cultured cardiomyocytes (1×10^6 cells/sample) were initially washed with HEPES buffer and then stained with 5 μmol/L X-rhod-1 AM for 30 min at room temperature. To avoid deesterification of intracellular Xrhod-1 AM in the cytosolic compartment, which would interfere with the detection of $[Ca^{2+}]$m, the cardiomyocytes were rinsed and incubated with 100 μM $MnCl_2$- HEPES for an additional 20 min to quench the cytosolic Ca^{2+} signal [16]. Fluorescence measurement was determined using a fluorescence plate reader (CytoFluor II; PerSeptive Biosystems; Framingham, MA) at an excitation wavelength of 580 nm and an emission wavelength of 645 nm for $[Ca^{2+}]$m. The images from the slides were captured using a digital camera connected to Image- Pro Plus software (Media Cybernetics; Silver Spring, MD).

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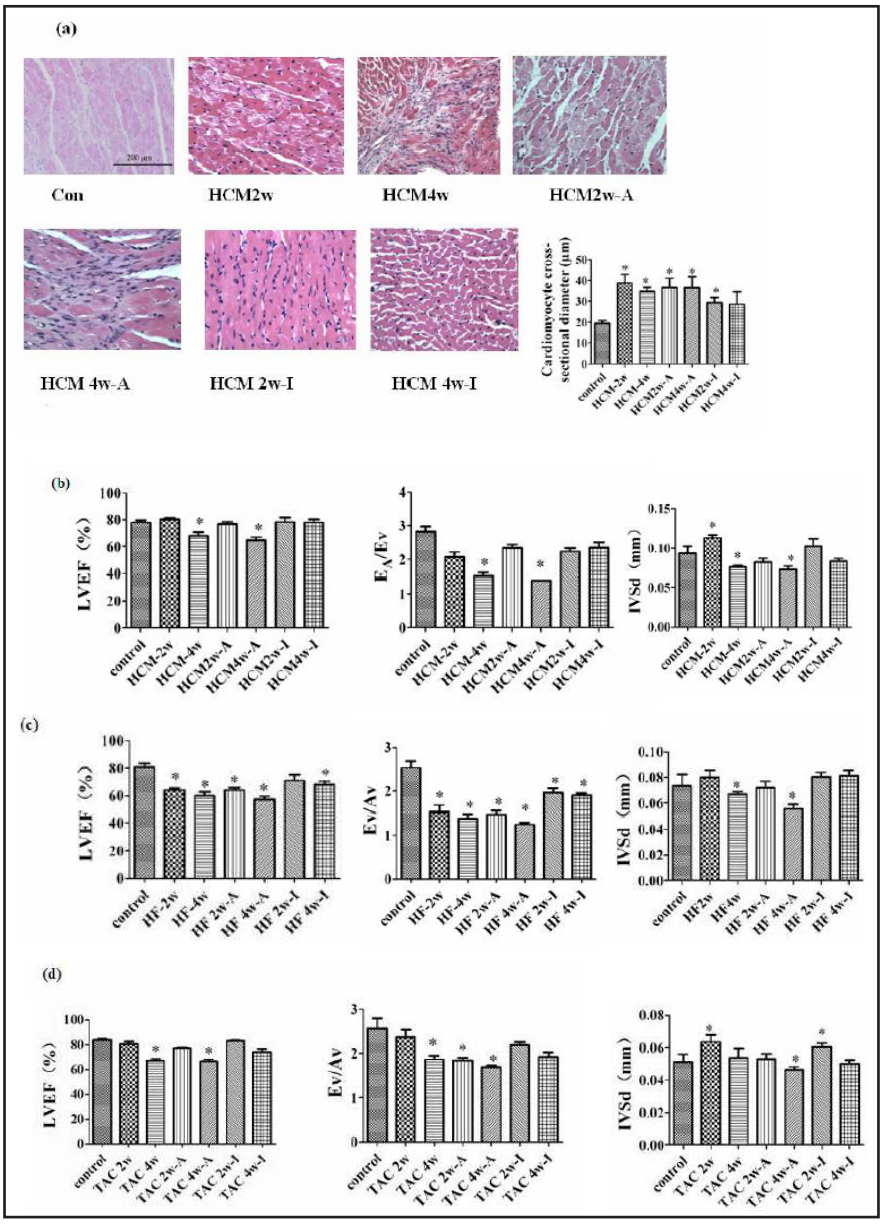

Fig. 1. Involvement of CaR in the functional changes of failing hearts. (a) HE staining of rat hearts and cardiomyocyte cross-sectional diameter (n=4). *p<0.05 vs. control group. (b-d) CaR induced changes in myocardial function in the failing hearts. Analysis of the left ventricular ejection fraction (LVEF) and the ratio of the early and atrial peak filling velocities (Ev/Av) showed that the cardiac systolic and diastolic function was improved in the HCM 4w, HCM 4w-A, HF 2w, HF 4w, HF 4w-A, TAC 4w and TAC 4w-A groups. Analysis of the interventricular septal diastolic thickness (IVSd) showed that the thickness of anterior lateral and interventricular septal walls was increased in the HCM 2w, HCM 4w, HCM 2w-A, HCM 2w-I, HCM 4w-I, TAC 2w and TAC 2w-I groups.Data are presented as the means ± SEM (n =6-8 per group). *p < 0.05 versus control group.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (Δψm) was measured with a unique cationic dye of 5,5‘,6,6‘-tetrachloro 1,1‘3,3‘-tetraethylbenzimidazolcarbocyanine iodide (JC-1), as previously described [18]. Briefly, cells were seeded on culture slides and treated according to experimental protocols. Cells were stained with JC-1 (1 μg/ml) at 37°C for 15 min and then rinsed three times with PBS. Observations were immediately made using a laser confocal scanning microscope. The ratio of JC-1 aggregate (red) to monomer (green) intensity was calculated.

Statistical Analysis

All values are expressed as mean ± S.E. unless noted otherwise. Differences between data groups were evaluated for significance using Student t-test of unpaired data or one-way analysis of variance (ANOVA). P<0.05 was accepted as statistically significant.

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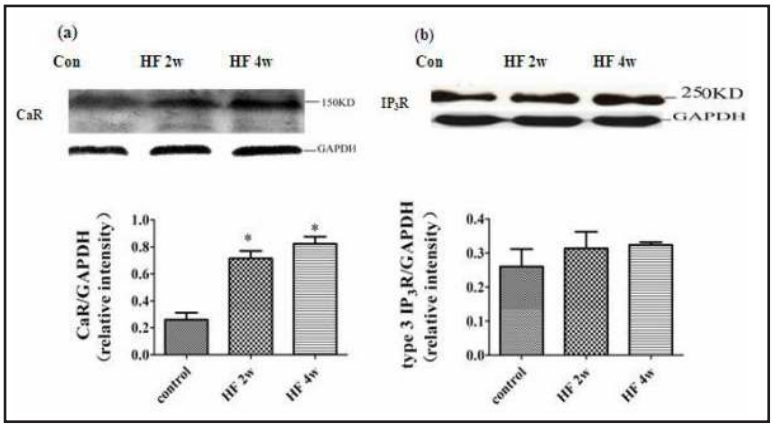

Fig. 2. CaR (a) and type 3 IP_3R (b) expression in rats with heart failure. Western blot and quantitative analysis of CaR and type 3 IP_3R expression in Iso animals. GAPDH was used as an internal control. Data are presented as the means ± SEM (n = 4-6 per group). *p < 0.05 versus control group.

Results

Effects of CaR activation on myocardial function during cardiac hypertrophy and heart failure

To investigate the *in vivo* effect of CaR on ER stress in failing hearts, we first established a cardiac hypertrophy model by injecting the rats with a high dose of Iso for 4 days. Morphological analyses showed that Iso induced myocyte hypertrophy, i.e., significantly increased the cardiomyocyte cross-sectional diameter in the HCM 2w and 4w and HCM 2w-A and 4w-A groups, respectively, and this parameter was significantly reduced in the hearts of rats treated with the CaR inhibitor calhex231 (Fig. 1a). Myocardial function was examined by echocardiography. Compared to the sham group, the interventricular septal diastolic thickness (IVSd) was increased in HCM 2w group. Increases in the left ventricular ejection fraction (LVEF) and the ratio between the early and atrial peak filling velocities (Ev/Av) showed that cardiac systolic and diastolic function was improved in the HCM 2w and HCM 2w-A groups. However, the LVEF and Ev/Av were decreased after Iso administration for 4 weeks compared to the sham group (Fig. 1b).

In contrast, 6 weeks after Iso administration, the LVEF and Ev/Av were decreased up to 60%, indicating the occurrence of heart failure. In the HF 2w, HF 4w, HF 2w-A and HF 4w-A groups, the LVEF and Ev/Av were significantly decreased compared to those in the HF-I 2w and 4w groups. These data suggest that CaR could be involved in cardiac hypertrophy and aggregated heart function in rats with heart failure (Fig. 1c).

We then created another animal model of heart failure by performing TAC and concurrently treated them with or without the CaR activator calindol or the CaR inhibitor calhex231 for 2 or 4 weeks. Similar results were observed from these TAC mice. TAC and TAC with calindol treatment for 2 weeks induced significant cardiac hypertrophy. The left ventricle function of the TAC 2w and TAC 2w-I and TAC 4w-I animals was slightly improved, as assessed by echocardiography, compared to the control and TAC 2w-A, TAC 4w, and TAC 4w-A groups (Fig. 1d).

We also noticed that the cardiomyocyte expression of CaR was significantly increased in the progression of heart failure (Fig. 2a). However, type 3 IP_3R expression was not changed significantly during heart failure induced by Iso (Fig. 2b).

Involvement of CaR in ER stress in hypertrophic and failing hearts in rats

To explore the *in vivo* effect of CaR on ER stress in failing hearts, we detected the expression of several molecular indicators of ER stress in hypertrophic and failing hearts in rats. ER stress responses were observed in the process of cardiac hypertrophy and heart failure, as assessed by the increased expression of GRP78 in the HCM, HCM-A, HCM 2w-I, HF and HF-A groups. The CaR inhibitor calhex231 significantly suppressed ER stress responses in HCM4w-I and HF4w-I groups (Fig. 3a,c). Similar results were also found in TAC mice. GRP78 expression in the TAC and TAC-A groups was increased at both 2 and 4 weeks (Fig. 3b).

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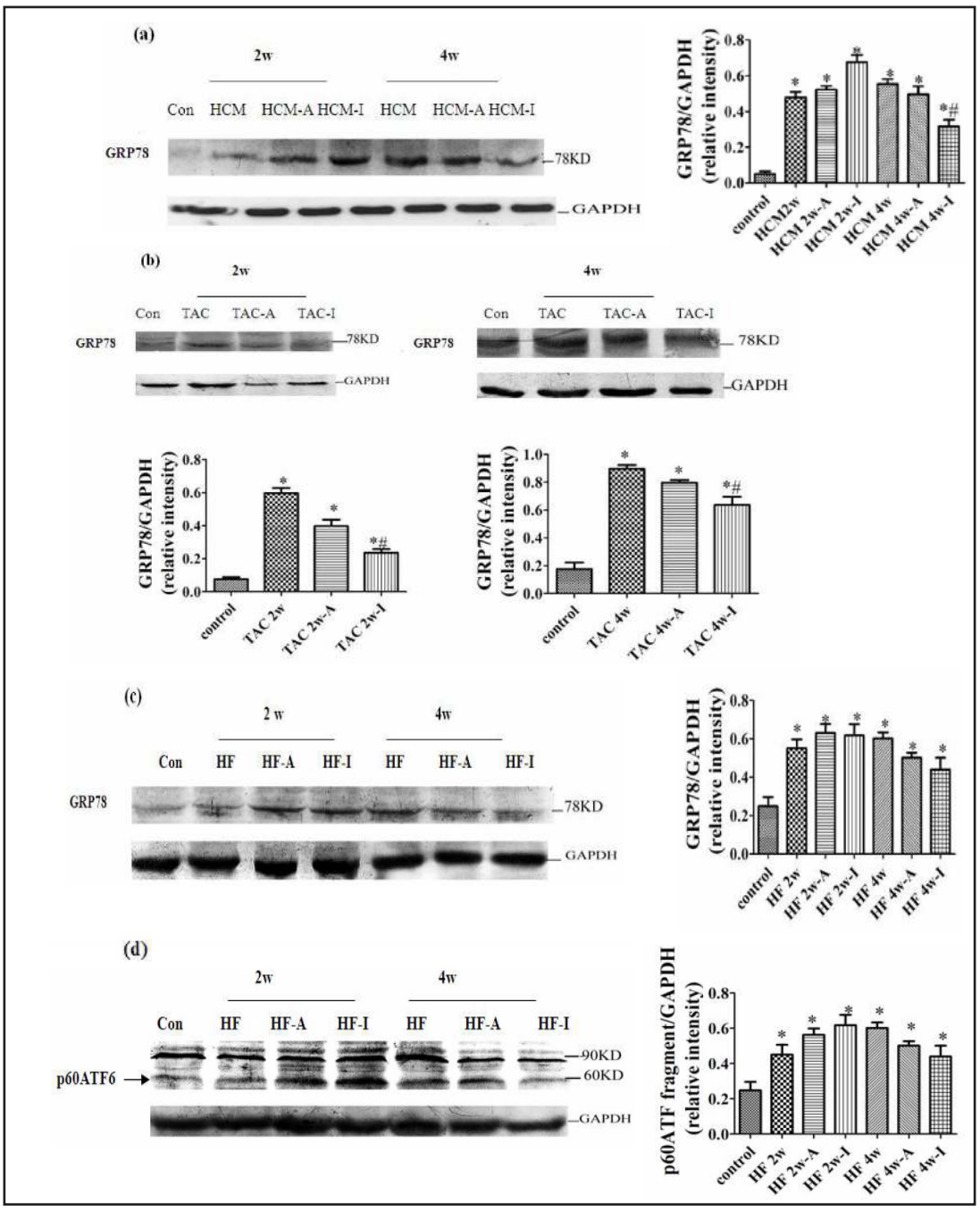

Fig. 3. CaR induced ER stress in failing hearts in rats and mice. ER chaperone GRP78 expression was increased in Iso (a) and TAC (b) animals with calindol-induced cardiac hypertrophy at 2 and 4 weeks, and treatment with calhex231 reduced the expression of GRP78. The expression of GRP78 (c) and ATF6 (d) in animals with heart failure induced by Iso were increased in groups treated with Iso and with Iso plus calindol. Protein levels were normalized to GAPDH (n=4-5). *p<0.05 vs. sham, #p<0.05 vs. HF 4w.

The ATF6 is a transcription factor that is retained in the ER with GRP78. When unfolded proteins are accumulated, GRP78 is sequestered away, resulting in the translocation of ATF6 to the Golgi apparatus, where it is cleaved and activated. A 60-kDa form of ATF6 (p60ATF6) is released into the cytoplasm, and a 36-kDa form of this protein (p36ATF6) is translocated to the nucleus. Western blot analysis showed that p60ATF6 was significantly increased in

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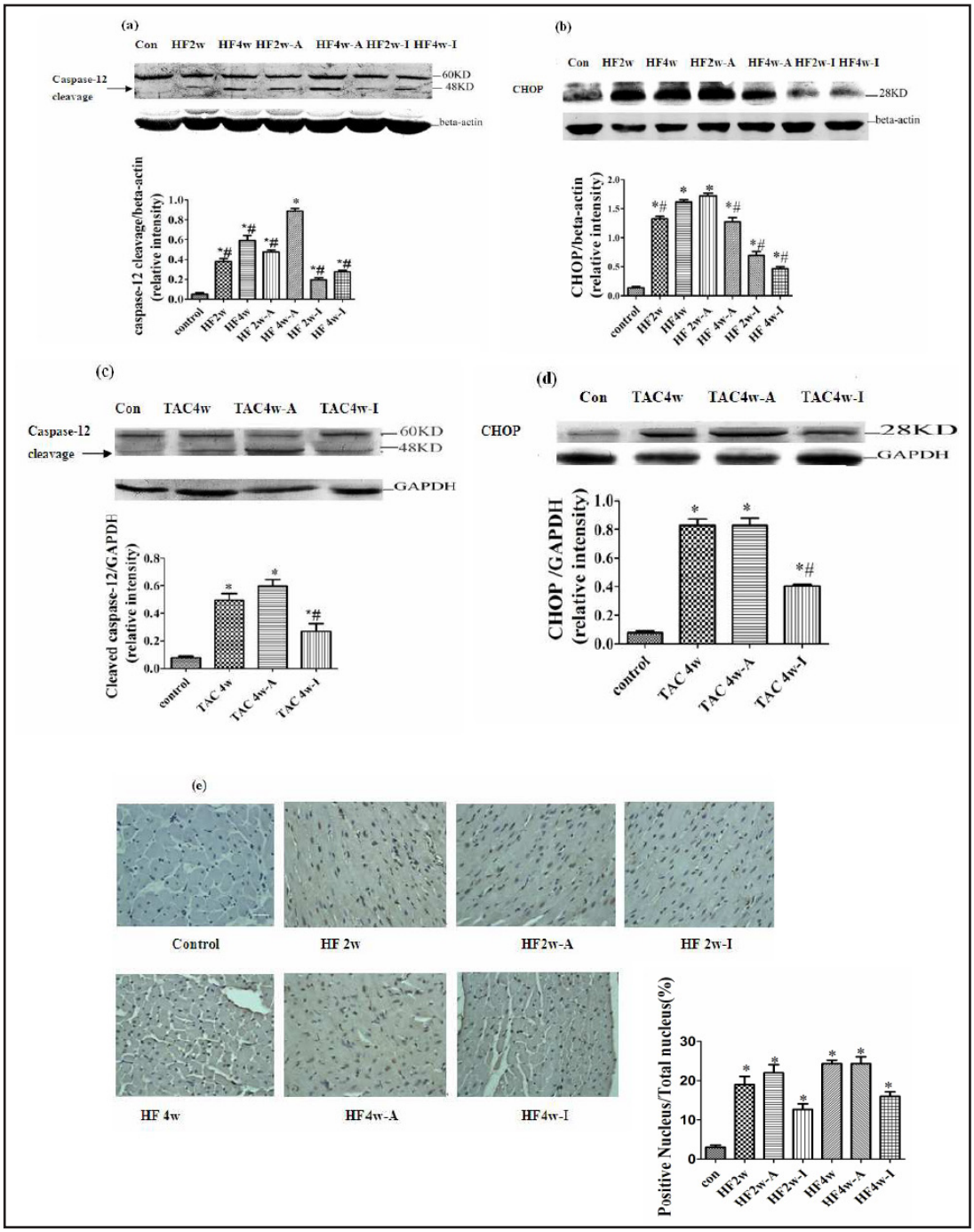

Fig. 4. CaR initiated ER stress-mediated apoptosis in animals with heart failure. Cleaved caspase-12 (a) and CHOP (b) expression was increased in animals treated with Iso and with Iso plus calindol at 2 and 4 weeks. The expression of cleaved caspase-12 (c) and CHOP (d) was increased in animals treated with TAC and with TAC plus calindol at 4 weeks. The levels of cleaved caspase-12 and CHOP were normalized to beta-actin or GAPDH (n=4-5). *p<0.05 vs. sham, #p<0.05 vs. HF 4w or TAC 4w. (e) Representative images of TUNEL staining for the different experimental groups. Nuclei with brown staining indicate TUNEL-positive cells. Scale bar : 50μm. Values are the means ± SEM (n = 6). *p<0.05 vs. sham, #p<0.05 vs. HF 4w.

the HF 4w and HF 2w-A animals compared with the HF 4w-A, HF 2w-I and HF 4w-I groups (Fig. 3d).

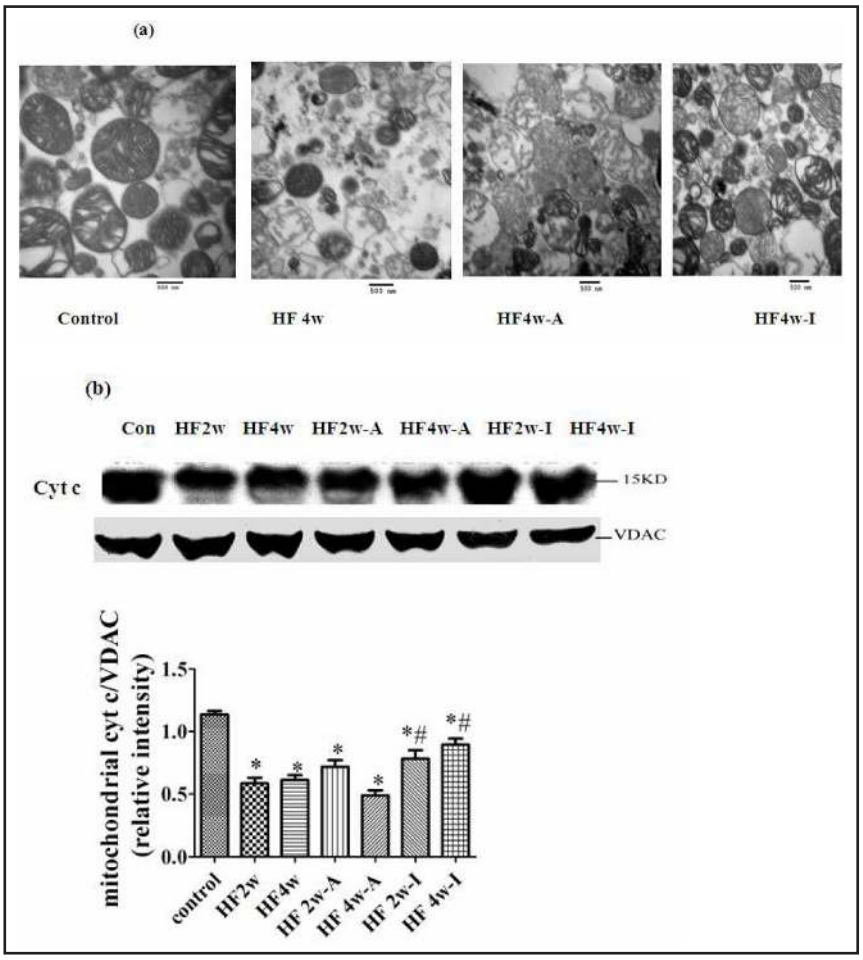

Fig. 5. CaR induced morphological and functional changes in the mitochondria of rats with heart failure. (a) Ultrastructural changes in the mitochondria of rats with heart failure at 4 weeks, as viewed by EM (×20000) (n=3). (b) Leakage of cytochrome *c* from the mitochondria to the cytoplasm, as determined by Western blot. The fold changes for the cyt c values are means ± SEM (n= 3-4). *p < 0.05 vs. control, #p < 0.05 vs. HF 4w.

CaR activation enhanced ER stress-mediated apoptosis in hypertrophic and failing hearts in rats

Caspase-12 and CHOP are characteristic markers of ER stress-induced apoptosis [20, 21]. We examined the expression of caspase-12 and CHOP in hearts subjected to Iso and either calindol or calhex231. Our results showed that the expression of CHOP and cleavage of caspase-12 increased dramatically in the HF 4w and HF 4w-A groups. Inhibition of CaR abolished the expression of CHOP and cleavage of caspase-12, while activation of CaR increased their expression (Fig. 4a, b). In accordance with the above results, the level of apoptotic cardiomyocytes, as examined by TUNEL, markedly increased in the HF 2w, HF 4w, HF2w -A, and HF 4w-A groups compared to the HF 2w-I and HF 4w-I groups (Fig. 4e).

The cleavage of caspase-12 and CHOP expression levels were significantly increased in the TAC 4w and TAC 4w-A groups compared to the control and TAC 4w-I groups (Fig. 4c,d). Taken together, CaR induces sustained ER stress, which initiates its apoptotic pathway.

Morphological changes in the ultrastructure and cytochrome c content of mitochondria in heart failure

In the sham group, mitochondria showed regular cristae and a normal arrangement of sarcomeres. In the HF 4w and HF-A 4w groups, mitochondrial derangements were always present. The mitochondria appeared swollen, with disorganized cristae and the formation of wrinkled bodies, and some mitochondria had a ruptured outer membrane. The mitochondria in the HF-I 4w group had slightly swollen mitochondria and disorganized cristae (Fig. 5a). The mitochondrial release of cytochrome *c* was analyzed to prove the involvement of the mitochondrial apoptotic pathway in heart failure. We found that the release of cytochrome *c*

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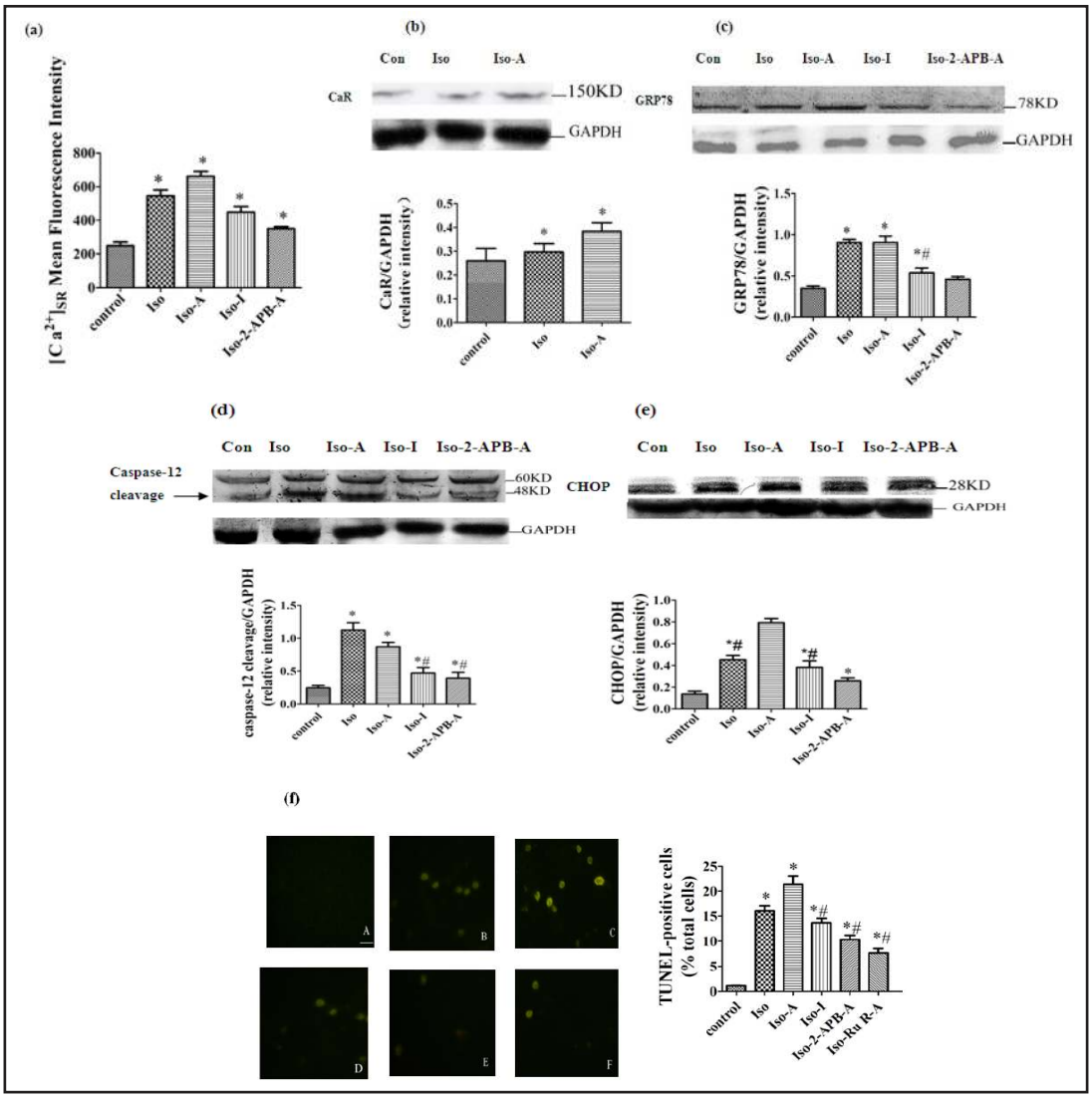

Fig. 6. CaR activation induced Ca^{2+} release from the SR as well as ER stress and ER stress-mediated apoptosis in NVCMs treated with Iso. (a) CaR activation induced Ca^{2+} release from the SR after Iso administration. The changes of fluorescence intensity, which corresponded to calcium concentration, in the SR were measured by Fluo-5N staining. (b) Treatment with Iso and Iso plus calindol increased CaR expression in NVCMs(n=4-5). *p<0.05 vs. control. (c) CaR activation promoted ER stress in NVCMs treated with Iso (n=4-5). (d-e) CaR increased the expression of cleaved caspase-12 (d) and CHOP (e) in NVCMs. Cells were treated with Iso or Iso plus calindol for 48 hours. Cell lysates were then immunoblotted for caspase-12 and CHOP. Cleaved caspase-12 and CHOP expression levels were normalized to GAPDH (n=4-5). (f) TUNEL-stained nuclei of apoptotic myocytes were analyzed morphologically, and the data are expressed as the percentage of total nuclei (n=5) (A) control, (B) Iso, (C) Iso-A, (D) Iso-I, (E) Iso-2-APB-A, (F) Iso-RuR-A. (magnification × 400) Scale bar : 20μm. *p<0.05 vs. control, #p < 0.05 vs. Iso.

from the mitochondria in the HF 2w, HF 4w, HF 2w-A, and HF 4w-A groups was significantly decreased compared with the control, HF 2w-I, and HF 4w-I groups (Fig. 5b).

Activation of CaR depleted $[Ca^{2+}]_{SR}$ in rat neonatal ventricular cardiomyocytes treated with Iso

To further investigate the induction of the ER stress-mediated apoptotic pathway by CaR, we examined $[Ca^{2+}]_{SR}$ by Fluo-5N staining in the rat neonatal ventricular cardiomyo-

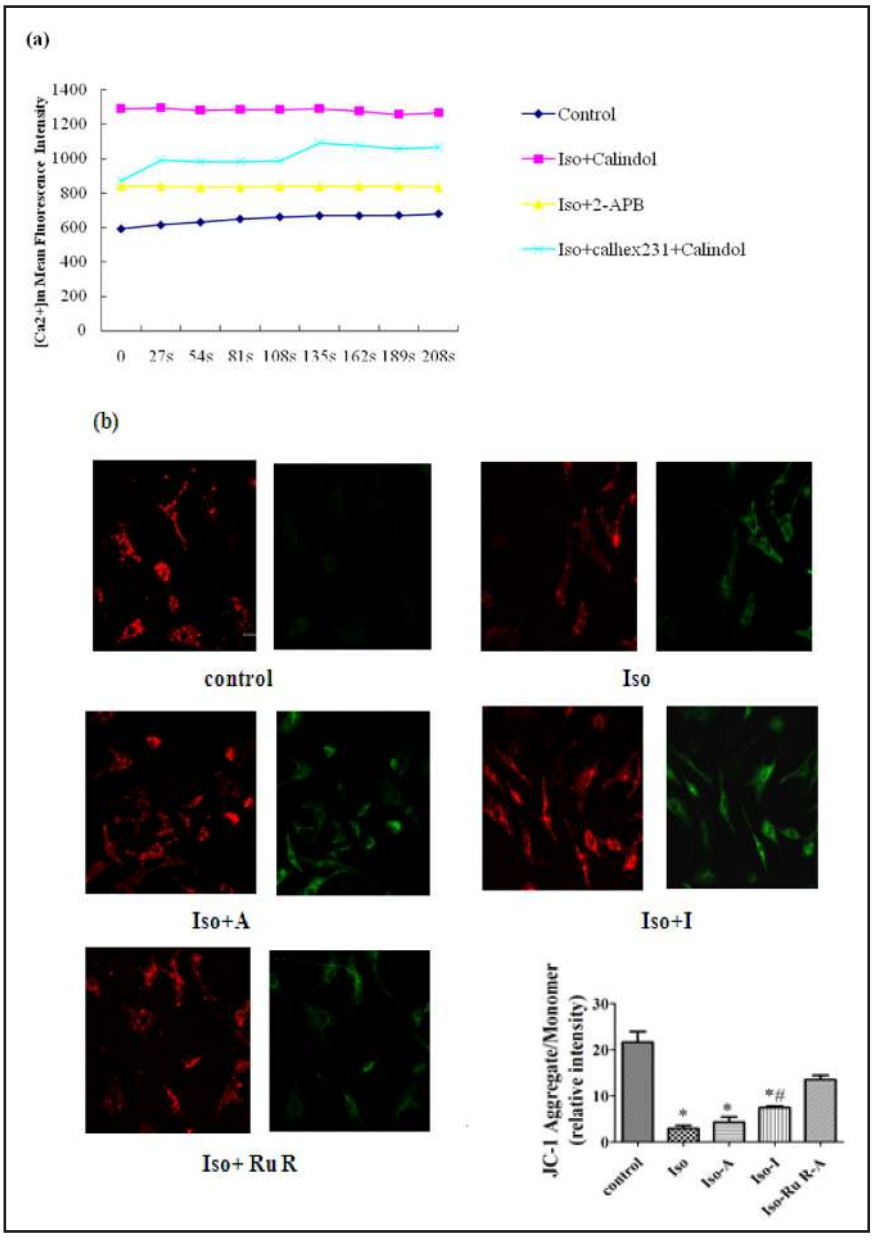

Fig. 7. CaR activation induced the mitochondrial apoptosis pathway. (a) Measurement of $[Ca^{2+}]m$ after administration of Iso for 48 hours by laser confocal microscopy. The changes in fluorescence intensity, which corresponded to the calcium concentration, in the mitochondria were measured by x-rhod-1AM staining. (b) Effect of CaR activation on Δψm in NVCMs after administration of Iso for 48 hours by laser confocal microscopy. Summarized data for the relative changes of JC-1 fluorescence. Scale bar : 20μm. (c) The release of cytochrome *c* from mitochondria in NVCMs (n=4-5). The data are the means ± SEM. * p<0.05 vs. control, #p < 0.05 vs. Iso.

cytes (NVCMs). Fluo-5N is a low-affinity Ca^{2+} indicator (Kd = 400 μmol/L), which is only bright where $[Ca^{2+}]$ is very high, such as in the SR [22]. NVCMs were loaded with Fluo-5N and permeabilized with saponin. After treatment with Iso for 48 h, the Fluo-5N signal was detected in the SR. We found that the fluorescence intensity in the SR in the Iso-A group was significantly decreased compared to the control, Iso plus calhex231, 2-APB (IP_3Rs inhibitor) groups. Some studies have confirmed that 2-APB inhibits IP_3Rs and prevents the PE-induced enhancement of Ca^{2+} sparks in neonatal cardiomyocytes [23]. Our study also suggests that 3 μM 2-APB maintains $[Ca^{2+}]_{SR}$ through the inhibition of Ca^{2+} release from the SR via IP_3Rs. These results suggest that the activation of CaR by calindol induces the release of Ca^{2+} from the SR (Fig. 6a).

Expression of CaR and its involvement in ER stress in neonatal ventricular cardiomyocytes

We found that CaR expression was increased in the Iso and Iso-A groups compared to the control group (Fig. 6b). To investigate whether CaR participates in ER stress, NVCMs were pretreated with Iso and calindol. We found that the expression of GRP78 was signifi-

cantly increased; however, pretreatment of the NVCMs with calhex231 before Iso treatment had little effect on GRP78 expression (Fig. 6c).

CaR induced ER stress-mediated cardiomyocyte apoptosis

As shown Fig. 6d and e, the levels of cleaved caspase-12 and CHOP were increased by CaR activation in NVCMs treated with Iso, whereas they were reduced by CaR inhibition. In accordance with these results, pretreatment of the cells with calhex231 significantly reduced the cell apoptosis induced by Iso, as determined by TUNEL. These results indicate that the activation of CaR increases ER stress responses and subsequent apoptosis in cardiomyocytes (Fig. 6f).

CaR activation increased $[Ca^{2+}]_m$ and reduced the mitochondrial membrane potential in neonatal rat ventricular cardiomyocytes (NVCMs) treated with Iso

Although calindol-activated CaR significantly reduced $[Ca^{2+}]_{SR}$, the role of type 3 IP_3Rs in the MAM in mediating Ca^{2+} uptake by mitochondria is less clear. To address this question, $[Ca^{2+}]_m$ was measured by X-rhod-1 AM staining after pretreatment with Iso for 48 h. $[Ca^{2+}]_m$ was markedly low in the control group. $[Ca^{2+}]_m$ was significantly higher in the Iso and Iso-A groups than in the Iso-I and Iso-Ru R groups (Fig. 7a). The mitochondrial membrane potential was detected with JC-1 staining. The ratio of the intensity of JC-1 aggregates (red) to monomers (green) was reduced in the Iso and Iso-A groups compared with the control, Iso-I, and Iso-Ru R groups (Fig. 7b).

To demonstrate that $[Ca^{2+}]_{SR}$ depletion induced by CaR activation causes apoptosis via a mitochondrial mediated pathway, the mitochondrial release of cytochrome *c* was analyzed to prove the involvement of the mitochondrial apoptotic pathway. The level of cytochrome *c* in the mitochondria in the Iso and Iso-A groups was significantly decreased compared with the control (1.0 ± 0.1), Iso-I, and Iso-Ru R groups (Fig. 7c).

Discussion

The present study describes the role of CaR in inducing SR- and mitochondria-dependent apoptosis in heart failure. This role appears to be dependent on the anatomic functional interplay between the SR and mitochondria and involves Ca^{2+} transfer in these two organelles. Our study shows that the activation of CaR induces the release of Ca^{2+} from the SR, which causes ER stress and apoptosis. Simultaneously, an increase in Ca^{2+} uptake into the mitochondria through the MAM causes an increase in $[Ca^{2+}]_m$ and initiates the mitochondrial apoptotic pathway in the failing heart.

Heart failure is characterized by a decline in the force of contraction and dysregulation in intracellular Ca^{2+} homeostasis within myocytes [24]. Under physiological conditions, Ca^{2+} enters cardiomyocytes through the L-type Ca^{2+} channel (LTCC) and β-adrenergic receptor (β-AR), which causes the release of a large amount of Ca^{2+} from the SR by activating the RyR, leading to contraction. However, it has been confirmed that this Ca^{2+} influx is induced by a persistent increase of intracellular calcium concentration through LTCC and the activation of β-AR in failing hearts, which can lead to cellular necrosis and apoptosis [25]. Whether CaR plays a role in this process remains unclear. CaR, a G protein-coupled receptor, initiates complicated intracellular signals through the modulation of a series of intracellular signaling proteins, including G proteins and PLC, and in turn, stimulates IP_3 production and promotes intracellular Ca^{2+} release. CaR is important for the maintenance and regulation calcium homeostasis [26]. Although CaR is also widely expressed in tissues involved in calcium homeostasis, it modulates various cellular functions, including peptide secretion, ion channel/transporter activity, gene expression, proliferation, differentiation and apoptosis [27]. In our study, we chose calindol, a specific agonist of CaR, and calhex231, a specific inhibitor of CaR, to regulate CaR activity and confirm the role of CaR in failing hearts. Our data show that

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the expression of CaR in the hearts of rats subjected to Iso and calindol was significantly increased. These results reveal that CaR could be involved in the development of heart failure.

Although the main mechanism of excitation-contraction coupling in cardiac muscle is calcium-induced calcium release via RyR, previous studies have revealed that IP_3Rs also play a role in IP_3-induced calcium release. IP_3Rs are ligand-gated Ca^{2+} channels that function to release Ca^{2+} in response to IP_3 [28, 29]. Moreover, some evidence has demonstrated that IP_3R expression in the ventricles of patients with end-stage chronic heart failure is upregulated when compared to that in normal patients, in contrast to RyR, which is down-regulated [30]. However, our results have not found that type 3 IP_3R expression is up-regulated in the failing hearts, which may be due to our heart failure model not in the end stage.

Abnormal intracellular Ca^{2+} cycling and SR function are the main reasons leading to failing cardiomyocytes [31, 32]. Because the function of SR-resident chaperones is Ca^{2+}-dependent, the accumulation of unfolded proteins and aberrant Ca^{2+} regulation in the SR lead to the activation of ER stress [33]. Although some studies have implicated that ER stress induces heart failure, how ER stress is initiated in failing hearts in still unknown. Our present study suggests that CaR activation may play an important role in ER stress in cardiac hypertrophy and failure. Therefore, we analyzed the expression of ER stress-related proteins in our Iso and calindol groups as well as in our TAC model. While the initial responses to ER stress may be adaptive, leading to an elevation in the levels of classic ER chaperones, such as GRP78 and p60ATF6, prolonged ER stress could initiate the apoptotic signaling pathway, inducing CHOP, JNK and caspase-12 activation [34, 35]. Some studies have shown that cytosolic Ca^{2+} were released from ER and lead to the activation of several transcription factors, such as CHOP, PERK and elf2, essential components ER stress-mediated apoptosis [36, 37]. Caspase-12 has been shown to be located at the cytosolic side of the ER membrane and to be actively cleaved in response to ER stress, caspase-12 is the only known substrate to caspase-12 [38].We found that the expression of GRP78 and p60ATF6 is up-regulated in cardiac hypertrophy and failure. CHOP and cleavage of caspase-12 expression levels were increased only in the heart failure groups and with the treatment of calindol, whereas theses expression levels were markedly lower in the heart failure groups treated with the CaR inhibitor. A strong ER stress response was also found in isoproterenol-stimulated neonatal ventricular cardiomyocytes (NVCMs), and the expression of GRP78 and caspase-12 was significantly higher in animals treated with Iso and with Iso plus calindol than in animals treated with Iso plus calhex231p. Taken together, these results suggest that the activation of CaR induces ER stress and associated apoptosis in heart failure. To further demonstrate the mechanism of activation of CaR that is involved in ER stress-mediated apoptosis, $[Ca^{2+}]_{SR}$ was examined with Fluo-5N in NVCMs. Our data showed that $[Ca^{2+}]_{SR}$ was significantly reduced upon CaR activation with calindol. $[Ca^{2+}]_{SR}$ was maintained with the administration of calhex231, 2-APB and U73122, respectively. These results demonstrate that CaR-induced Ca^{2+} release from the SR is a PLC-IP_3-dependent process.

It has been demonstrated that the mitochondria and SR form an interconnected network with a restricted number of close contacts, which is called the mitochondria-associated membrane (MAM) [8]. Ca^{2+} accumulates in the local MAM, where high concentration Ca^{2+} triggers the mitochondrial calcium uniporter (MCU) in mitochondria to induce an increase of Ca^{2+} uptake in mitochondria [39]. IP_3Rs play an important role in the macromolecular complexes on the surface of the SR membranes and promote Ca^{2+} release from the SR. The transport of Ca^{2+} through MCU can be stimulated with Gq-coupled receptor agonists, which induce IP_3 production and the subsequent release of Ca^{2+} from the SR, which causes a rapid rise in $[Ca^{2+}]_m$ [40, 41]. Thus, we postulate that CaR may be involved in promoting Ca^{2+} release from the SR and subsequently in the increase in $[Ca^{2+}]_m$. Therefore, we analyzed $[Ca^{2+}]_m$ using x-rhod-1 AM and found that $[Ca^{2+}]$m was elevated in the Iso and Iso plus calindol groups, whereas $[Ca^{2+}]_m$ was maintained in the Iso plus calhex231 group. The rapid mitochondrial Ca^{2+} uptake is related to the low affinity of the Ca^{2+} transport system. Ruthenium red, an inhibitor of the MCU, was also used in this experiment. The results revealed that $[Ca^{2+}]_m$ was maintained in the Iso-RuR group.

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SR-mitochondrial Ca^{2+} cross-talk may play a critical role in heart failure as an inducer of programmed cardiomyocyte death. Ca^{2+} transportation to the mitochondria plays a key role in initiating apoptosis by opening the mitochondrial permeability transition pore (mPTP) [42, 43]. These results suggest that both the SR and the mitochondria orchestrate the regulation of Ca^{2+} signaling between these two organelles. It has been reported that $[Ca^{2+}]_{SR}$ depletion causes bax- and bak-mediated permeability of the outer mitochondrial membrane, thereby releasing pro-apoptotic factors, particularly cytochrome *c* [44, 45]. Stimulation of these pathways triggers the translocation of death-promoting proteins (e.g., cytochrome *c*) from the mitochondria to the cytoplasm [46]. Our present data show that CaR activation induced cytochrome *c* release from the mitochondria during heart failure. Morphological analysis of apoptosis in cardiomyocytes was also performed using EM in the present study, and the results showed that CaR activation caused aggregated mitochondrial derangements, such as swelling, disorganized cristae and loss of normal striations. TUNEL assay also showed that the activation of CaR increased the number of apoptotic cardiomyocytes. Thus, CaR causes the cardiomyocyte apoptosis related to the interplay between the SR and mitochondria in the progression of heart failure.

In conclusion, our results are the first to report that CaR plays an important role in SR-mitochondrial inter-organelle Ca^{2+} signaling through IP_3Rs, which are also involved in apoptosis during heart failure. We propose that CaR could be a valuable pharmacological target for heart failure therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Reference

1 Jiang H, Ge J: Epidemiology and clinical management of cardiomyopathies and heart failure in China. Heart 2009;95:1727–31.
2 Orrenius S, Zhivotovsky B, Nicotera P: Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 2003;4:552–565.
3 Schroder M, Kaufman RJ: The mammalian unfolded protein response. Annu Rev Biochem 2005;74:739–789.
4 Kaufman RJ: Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002;110:1389–1398.
5 Ni L, Zhou CQ, Duan QL, Lv JG, Fu XN, Xia Y, Wang DW: β-AR Blockers Suppresses ER Stress in Cardiac Hypertrophy and Heart Failure. PLoS ONE 2011;6: 27294-27304
6 Murgia M, Giorgi C, Pinton P, Rizzuto R: Controlling metabolism and cell death: at the heart of mitochondrial calcium signalling. J Mol Cell Cardiol 2009;46:781–788.
7 Lukyanenko V, Chikando A, Lederer WJ: Mitochondria in cardiomyocyte Ca^{2+} signaling. Int J Biochem Cell Biol 2009;41:1957–1971.
8 Hayashi T, Su TP: Sigma-1 receptor chaperones at the ER mitochondrion interface regulate Ca^{2+} signaling and cell survive. Cell 2007;131:596-610.
9 Dorn Gerald, Scorrano Luca: Two Close, Too Close Sarcoplasmic Reticulum–Mitochondrial Crosstalk and Cardiomyocyte Fate Circ Res 2010;17:689-699.

10 Jimbo A, Fujita E, Kouroku Y, Ohnishi J, Inohara N, Kuida K, Sakamaki K, Yonehara S, Momoi T: ER stress induces caspase-8 activation, stimulating cytochrome c release and caspase-9 activation. Exp Cell Res 2003;283:156–166.
11 Chami M, Oules B, Szabadkai G, Tacine R, Rizzuto R, Paterlini-Brechot P: Role of SERCA1 truncated isoform in the proapoptotic calcium transfer from ER to mitochondria during ER stress. Mol Cell 2008;32:641–651
12 Garcia-Perez C, Hajnoczky G, Csordas G: Physical coupling supports the local Ca^{2+} transfer between sarcoplasmic reticulum subdomains and the mitochondria in heart muscle. J Biol Chem 2008;283:32771–32780
13 Brown EM, MacLeod RJ: Extracellular calcium sensing and extracellular calcium signaling. Physiol Rev 2001;81:239–297.
14 Zhang WH, Fu SB, Lu FH, Wu B, Gong DM, Pan ZW: Involvement of calcium-sensing receptor in ischemia / reperfusion induced apoptosis in rat cardiomyocytes. Biochem Biophys Res Commun 2006;347:872–881.
15 Krenek P, Kmecova J, Kucerova D, Bajuszova Z, Musil P: Isoproterenol-induced heart failure in the rat is associated with nitric oxidedependent functional alterations of cardiac function. Eur J Heart Fail 2009;11:140–146.
16 Marano G, Palazzesi S, Fadda A, Vergari A, Ferrari AU: Attenuation of aortic banding-induced cardiac hypertrophy by propranolol is independent of beta-adrenoceptor blockade. J Hypertens 2002;20:763–769
17 Sun HY, Wang NP, Halkos ME, Kerendi F, Kin H, Wang RX, Guyton RA, Zhao ZQ: Involvement of Na^+/H^+ exchanger in hypoxia/re-oxygenationinduced neonatal rat cardiomyocyte apoptosis. Eur J Pharmacol 2004;486:121-131.
18 Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, Guyton RA, Vinten-Johansen J, Zhao ZQ: Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca^{2+} overload. Am J Physiol Heart Circ Physiol 2005;288:H1900-H1908.
19 Lu FH, Tian ZL, Zhang WH, Zhao YJ, Li HL, Ren H, Zheng HS, Liu C, Hu GX, Tian Y, Yang BF, Wang R, Xu CQ: Calcium-sensing receptors regulate cardiomyocyte Ca^{2+} signaling via the sarcoplasmic reticulum-mitochondrion interface during hypoxia/reoxygenation J Biomed Sci 2010;17;17:50.
20 Dickhout JG, Carlisle RE, Austin RC. Interrelationship between cardiac hypertrophy, heart failure, and chronic kidney disease: endoplasmic reticulum stress as a mediator of pathogenesis. Circ Res 2011;108:629-642
21 Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA: Caspase-12 mediates endoplasmicreticulum-specific apoptosis and cytotoxicity by amyloid-b. Nature 2000;403:98–103.
22 Lu FH, Tian ZL, Zhang WH, Zhao YJ, Bai SZ, Ren H, Chen H, Yu X, Wang JX, Wang LN, Li H, Pan ZW, Tian Y, Yang BF, Wang R, Xu CQ: Calcium-sensing receptors induce apoptosis in rat cardiomyocytes via the endo(sarco) plasmic reticulum pathway during hypoxia/reoxygenation Basic Clini Pharmac Toxicol 2009;106, 396–405
23 Bootman MD, Missiaen L, Parys JB, De Smedt H, Casteels R: Control of inositol triphosphate-induced Ca^{2+} release by cytosolic Ca^{2+}. Biochem J 1995;306:445-451.
24 Dorn GW, Maack C: SR and mitochondria: Calcium cross-talk between kissing cousins. J Mol Cell Cardiol 2013;55:42-9.
25 Dorn GW II: Apoptotic and non-apoptotic programmed cardiomyocyte death in ventricular remodelling. Cardiovasc Res 2009;81:465–473.
26 Brown EM: Clinical lessons from the calcium-sensing receptor. Nat Clin Pract Endocrinol Metab 2007;3:122–133.
27 Brown EM, Hebert SC: A cloned Ca2+ sensing receptor: a mediator of direct effects of extracellular Ca^{2+} on renal function? J Am Soc Nephrol 1995;6:1530–1540.
28 Mackenzie L, Bootman MD, Laine M, Berridge MJ, Thuring J, Holmes A, Li WH, Lipp P: The role of inositol 1,4,5- triphosphate receptors in Ca^{2+} signaling and the generation arrhythmias in rat atrial myocytes. J Physiol 2002;87:457-409.
29 Chastre E, Khan F, Landegren U, Soderberg O, Bootman MD, Roderick HL: Phosphorylation of inositol 1,4,5-triphosphate receptors by protein kinase B/Akt inhibits Ca^{2+} release and apoptosis. Proc Natl Acad Sci USA 2008;105:2427-2432.
30 Moschella MC, Watras J, Handa KK, Fyfe BS, Marks AR: Differential regulation of two types of intracellular calcium release channels during end-stage heart failure J Clin Invest 1995;95;888-894.
31 Kaufman RJ: Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002;110:1389–1398.
32 Wu G, Long X, Marin-Garcia J: Adenoviral SERCA1 overexpression triggers an apoptotic response in cultured neonatal but not in adult rat cardiomyocytes. Mol Cell Biochem 2004;267:123–132.

33 Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HR: IRE1 endoplasmic reticulum load to secretary capacity by processing the XBP-1 mRNA. Nature 2002;415:92–96.
34 Ferri KF, Kroemer G: Organelle-specific initiation of cell death pathways. Nat Cell Biol 2001;3:E255–263.
35 Chen X, Zhang X, Kubo H, Harris DM, Mills GD, Moyer J, Berretta R, Potts ST, Marsh JD, Houser SR: Ca^{2+} influx-induced sarcoplasmic reticulum Ca^{2+}overload causes mitochondrial-dependent apoptosis in ventricular myocytes. Circ Res 2005;97:1009–1017.
36 Sano FD, Ferraro E, Tufi R, Achsel T, Piacentini M, Cecconi F: Endoplasmic reticulum stress induced apoptosis by an apoptosome-dependent but caspase 12- independent mechanism. J Biol Chem 2006;281:2693-2700.
37 Luciani DS, Gwiazda KS, Yang TL, Kalynyak TB, Bychkivska Y, Frey MH, Jeffrey KD, Sampaio AV, Underhill TM, Johnson JD: Roles of IP3R and RyR Ca^{2+} channels in endoplamic reticulum stress and β-cell Death. Diabetes 2009;58:422-432.
38 Hoyer-Hansen M, Jaattela M: Connecting endoplasmic reticulum stress to autophage by unfolded protein response and calcium. Cell Death Differ 2007;14:1576-1582.
39 Seguchi H, RitterM, Shizukuishi M, Ishida H, Chokoh G, Nakazawa H, Spitzer KW, Barry WH: Propagation of Ca^{2+} release in cardiac myocytes: role of mitochondria. Cell Calcium 2005;38:1–9.
40 Andrienko TN, Picht E, Bers DM: Mitochondrial free calcium regulation during sarcoplasmic reticulum calcium release in rat cardiac myocytes. J Mol Cell Cardiol 2009;46:1027–1036.
41 Belmonte S, Morad M: Shear fluid-induced Ca^{2+} release and the role of mitochondria in rat cardiomyocytes. Ann NY Acad Sci 2008;1123:5-15.
42 Garcia-Perez C, Hajnoczky G, Csordas G: Physical coupling supports the local Ca^{2+} transfer between sarcoplasmic reticulum subdomains and the mitochondria in heart muscle. J Biol Chem 2008;283:32771–32780.
43 Pacher P, Csordas G, Hajnoczky G: Mitochondrial Ca^{2+} signaling and cardiac apoptosis. Biol Signals Recept 2001;10:200–223.
44 Bathori G, Csordas G, Garcia-Perez C, Davies E, Hajnoczky G: Ca^{2+}-dependent control of the permeability properties of the mitochondrial outer membrane and voltage-dependent anion-selective channel (VDAC). J Biol Chem 2006;281:17347–17358.
45 Duchen MR: Role of mitochondria in health and disease. Diabetes 2004;53:S96-100.
46 Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW II, Robbins J, Molkentin JD: Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 2005;434:658–662.