Attenuation of the upregulation of NF-κB and AP-1 DNA-binding activities induced by tunicamycin or hypoxia/reoxygenation in neonatal rat cardiomyocytes by SERCA2a overexpression

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Abstract. The present study aimed to investigate the effects of the overexpression of sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2a) on endoplasmic reticulum (ER) stress (ERS)-associated inflammation in neonatal rat cardiomyocytes (NRCMs) induced by tunicamycin (TM) or hypoxia/reoxygenation (H/R). The optimal multiplicity of infection (MOI) was 2 pfu/cell. Neonatal Sprague-Dawley rat cardiomyocytes cultured \textit{in vitro} were infected with adenoviral vectors carrying SERCA2a or enhanced green fluorescent protein genes, the latter used as a control. At 48 h following gene transfer, the NRCMs were treated with TM (10 µg/ml) or subjected to H/R to induce ERS. The results of electrophoretic mobility shift assay (EMSA) revealed that overexpression of SERCA2a attenuated the upregulation of nuclear factor (NF)-κB and activator protein-1 (AP-1) DNA-binding activities induced by TM or H/R. Western blot analysis and semi-quantitative RT-PCR revealed that the overexpression of SERCA2a attenuated the activation of the inositol-requiring 1α (IRE1α) signaling pathway and ERS-associated apoptosis induced by TM. The overexpression of SERCA2a also decreased the level of phospho-p65 (Ser536) in the nucleus, as assessed by western blot analysis. However, the overexpression of SERCA2a induced the further nuclear translocation of NF-κB p65 and higher levels of tumor necrosis factor (TNF)-α transcripts in the NRCMs, indicating the occurrence of the ER overload response (EOR). Therefore, the overexpression of SERCA2a has a ‘double-edged sword’ effect on ERS-associated inflammation. On the one hand, it attenuates ERS and the activation of the IRE1α signaling pathway induced by TM, resulting in the attenuation of the upregulation of NF-κB and AP-1 DNA-binding activities in the nucleus, and on the other hand, it induces EOR, leading to the further nuclear translocation of NF-κB and the transcription of TNF-α. The preceding EOR may precondition the NRCMs against subsequent ERS induced by TM. Further studies using adult rat cardiomyocytes are required to prevent the interference of EOR. The findings of the present study may enhance the current understanding of the role of SERCA2a in cardiomyocytes.

Introduction

Heart failure (HF) is becoming an increasingly serious public health concern (1). Despite recent advances in treatment, HF remains a fatal clinical syndrome. In the mouse, rat and human adult heart, sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2a) is the major cardiac isoform,
which pumps Ca\(^{2+}\) from the cytosol to the sarcoplasmic reticulum (SR) lumen utilizing the energy obtained by hydrolyzing ATP. HF is associated with the decreased expression and activity of SERCA2a (2-4). For this reason, SERCA2a has become an attractive target for the gene targeted therapy of HF. The abnormal calcium flux, and contraction and relaxation of cardiomyocytes in a failing heart may be improved by the transfer of SERCA2a (5). The improvement in cardiac contractility following SERCA2a transfer has been confirmed in a number of small and large animal models of HF induced by pressure overload, volume overload, ischemia, rapid ventricular pacing, or long-term isoproterenol stimulation. In a porcine volume-overload HF model (6), rAAV1-mediated intracoronary gene transfer in vivo has been reported to maintain the contractile function and improve cardiac remodeling. In both transgenic mice and rats, the overexpression of SERCA2 has been shown to enhance calcium transients, myocardial contractility and the relaxation in the absence or presence of pressure overload (7-12). In addition to its beneficial effects on myocardial contractility, the transfer of SERCA2a revives energy metabolism in the heart (13-15), decreases the c a\(^{2+}\) leak from the SR (16), restores electrical stability (17), reduces arrhythmic aftercontractions (18), decreases ventricular arrhythmias (16,19,20), suppresses cellular alternans (21) and increases coronary flow by activating endothelial nitric oxide synthase in endothelial cells (22). Moreover, the calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) study demonstrated the safety of SERCA2a therapy in patients with advanced HF and unraveled the benefits of this therapy (23-25). However, in the CUPID 2 study (26), AAV1-SERCA2a did not improve the clinical course of HF.

Misfolded proteins in the endoplasmic reticulum (ER) can induce the unfolded protein response (UPR). The UPR is composed of at least three branches (27). In resting cells, the three ER-located stress sensors, namely double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), inositol-requiring 1a (IRE1a), and activating transcription factor (ATF)6, are associated with immunoglobulin heavy chain-binding protein (BiP) and are maintained in an inactive state. In response to ER stress (ERS), PERK phosphorylates the α subunit of the eukaryotic protein synthesis initiation factor 2 (eIF2α), resulting in the inhibition of translation of the majority of mRNAs, but allowing for the translation of ATF4 mRNA. Under ERS conditions, IRE1α autophosphorylates and activates its RNase activity, resulting in the splicing of X-box binding protein-1 (XBP1) mRNA and the production of an active spliced XBP1 isoform. In parallel, following its release from BiP, ATF6 migrates to the Golgi apparatus, where it is cleaved by site-1 protease (SIP) and site-2 protease (S2P). The functional cleaved fragment of ATF6 is then released and migrates to the nucleus. The UPR leads to apoptosis when cells fail to address the protein folding defects and cannot re-establish homeostasis in the ER.

It has been shown that the UPR and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) interact at multiple levels and are interconnected through the production of reactive oxygen species (ROS), the release of calcium ion from the ER, activation of NF-κB and c-Jun N-terminal kinases (JNK) and the induction of the acute-phase response (27). Under ERS conditions, the PERK-induced phosphorylation of eIF2α inhibits the translation of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor-α (IκBα), decreasing the export of nuclear NF-κB to the cytoplasm. In response to ERS, the phosphorylated cytoplasmic domain of IRE1α recruits tumor necrosis factor-α-receptor-associated factor 2 (TRAF2). The IRE1α-TRAF2 complex interacts with IκB kinase (IKK) and/or JNK to activate these kinases. Activated IKK activates NF-κB through phosphorylation of IκB, initiating the degradation of IκB. Activated JNK activates the transcription factor activator protein-1 (AP-1) through phosphorylation. Activated NF-κB and AP-1 translocate to the nucleus and induce the transcription of inflammation-related genes. ATF6 can activate NF-κB through the protein kinase B (Akt) pathway. In addition, the ERS-triggered release of calcium from the ER and ROS can activate NF-κB (28).

Liu et al (29) revealed that the cardiomycyte-specific tamoxifen-inducible disruption of SERCA2 induced ER/SR structural changes, UPR and apoptosis. As also previously demonstrated, in a porcine myocardial ischemia model, the overexpression of SERCA2a significantly attenuated the activation of UPR and decreased ERS-associated apoptosis (30).

In the above context, it was hypothesized that the overexpression of SERCA2a could attenuate ERS by maintaining calcium homeostasis, thereby attenuating ERS-associated inflammation. The present study was thus conducted to explore this premise by overexpressing SERCA2a in neonatal rat cardiomyocytes (NRCMs).

Materials and methods

Cell culture and experimental protocol. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011) of National Research Council (US) (31) and approved by the Institutional Animal Care and Use Committee (IACUC) of PLA General Hospital (approval no. 2013-x7-28). The NRCMs were isolated from 1-day-old Sprague-Dawley rats. Pups were anesthetized with 5% isoflurane and sacrificed by cervical dislocation. Hearts were removed and immediately placed in cold phosphate-buffered saline (NaCl 136.75 mmol/l, KCl 2.68 mmol/l, Na\(_2\)HPO\(_4\) 9.75 mmol/l, KH\(_2\)PO\(_4\) 1.47 mmol/l, glucose 5.50 mmol/l, pH 7.4). The ventricles were minced and digested with 0.15% trypsin for 6-10 min at 37°C, and the supernatant was then transferred to a centrifuge tube containing Dulbecco’s modified Eagle’s medium (cat. no. 31600-034; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Shandong Yin Xiang Wei Ye Group Co., Ltd.), 100 IU/ml penicillin, and 100 µg/ml streptomycin (cat. no. 15140-122; Thermo Fisher Scientific, Inc.). The digestion was repeated ~10 times. Following centrifugation for 10 min, the supernatant was aspirated off and the cell pellet was resuspended in complete culture medium. The suspended cells were plated and incubated in a 5% CO\(_2\), 37°C incubator for 1 h. Thereafter, the culture medium containing non-adherent cells was collected, and these enriched cardiomyocytes were seeded in cell culture flasks with 0.1 mmol/l 5-bromo-2-deoxyuridine added to the medium to inhibit fibroblast proliferation. After two days, the cardiomyocytes were trypsinized and counted; the aliquots of cardiomyocyte
Determination of the optimal multiplicity of infection (MOI).

O'donnell et al (32) found out that the exogenous expression of SERCA in the NRCMs reduced the viability of the cells, with cell floaters occurring even if the MOI was as low as 5 pfu/cell. The apoptotic index in myocytes infected with adenoviral vectors carrying the wild-type SERCA1 gene was 7% at 2 pfu/cell and 31% at 10 pfu/cell. The expression of exogenous SERCA and acceleration of Ca2+ transients could be achieved with minimal cell damage in rat myocytes when the MOI was in the range of 1 to 4 pfu/cell. O'Donnell et al (32) also performed in situ immunofluorescence staining with specific antibodies against the exogenous SERCA1. It was found out that SERCA1 was densely packed within sarco/endoplasmic reticulum even in apparently normal cells. Severe structural changes occurred in cytopathic cells. It should be highlighted that both wild-type SERCA and inactive SERCA mutant produced cytotoxic effects. Thus, the investigators proposed that the dense accumulation of SERCA within a very limited sarco/endoplasmic reticulum space will disturb membrane structure and function and perturb calcium homeostasis (32). Therefore, the present study decided to perform a titration test on the MOIs in the NRCMs transferred with rAd-SERCA2a, with the expression level of SERCA2a, cell viability and LDH in the cell culture supernatant evaluated. The present study hoped to determine a certain MOI value, at which the high expression of SERCA2a could be achieved, while the cytotoxicity would be minimized to prevent the impact on cell inflammation.

**Electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared using the NPProtein Extraction kit (Exprogen Biotechnologies, Inc.). The sequences of the probes used for the assay were as follows: Ds-Bio-NF-κB probe, Bio-5'-AGTGGAGGACTTTCAGGC3'-Bio; Ds-Bio-API1 probe, Bio-5'-CGCTTGAAGTGACGCCCAGAA3'-Bio; Ds-Bio-OCT1 probe, Bio-5'-TGTCGATAGCAATACCTGAA3'-Bio. EMSA was carried out using the BiotinLight™ Chemiluminescent EMSA kit (Exprogen Biotechnologies, Inc.) according to the instruction manual. Competition experiments with 100-fold excess of unlabeled probe used as a specific competitor were performed to confirm the specificity of protein-DNA binding. Antibodies against NF-κB p50 (cat. no. sc-1190), NF-κB p65 (cat. no. sc-372), c-Jun (cat. no. sc-1694), and c-Fos (cat. no. sc-52) were purchased from Santa Cruz Biotechnology, Inc. A total of 4 µl of undiluted antibodies were added to 15-µl binding reactions. The samples were incubated for 20 min at room temperature. The sample was electrophoresed on a 1% agarose gel in 0.5X Tris-borate-EDTA buffer at 120 V for 1.5 h, and then electrophoretically blotted onto a nylon membrane at 380 mA for 1 h. The membrane was cross-linked in a UV-light cross-linker (Analytik Jena AG) for 10 min, and the biotin-labeled DNA was detected by chemiluminescence.

**Western blot analysis.** Primary antibodies against BiP (cat. no. 3183; 1:1,000), phospho-PERK (cat. no. 3179; 1:1,000), PERK (cat. no. 3192; 1:1,000), phospho-eIF2α (cat. no. 3398; 1:1,000), eIF2α (cat. no. 9722; 1:1,000), phospho-NF-κB p65 (Ser536) (cat. no. 3033; 1:1,000), NF-κB p65 (cat. no. 8242; 1:1,000), SERCA2 (cat. no. 9580; 1:1,000) and histone H3 (cat. no. 4499; 1:2,000) were purchased from Cell Signaling Technology, Inc., those against phospho-IRE1 (cat. no. ab48187; 1:1,000) and caspase-12 (cat. no. ab62484; 1:500) were from Abcam, that against IRE1 (cat. no. NB100-2324; 1:1,000) was from Novus Biologicals, LLC, that against CHOP (cat. no. sc-7351; 1:200) was from Santa Cruz Biotechnology, Inc. and that against GAPDH (cat. no. 60004-1-lg; 1:2,000) was from Proteintech Group, Inc. HRP-conjugated secondary antibodies of goat anti-mouse IgG (cat. no. sc-2005; 1:3,000)
and goat anti-rabbit IgG (cat. no. sc-2004; 1:3,000) were purchased from Santa Cruz Biotechnology, Inc. Whole-cell extracts were prepared using radioimmunoprecipitation assay lysis buffer (cat. no. CW2333; Beijing Cowinbiocience Co., Ltd.) containing a protease inhibitor cocktail (cat. no. CW2200; Beijing Cowinbiocience Co., Ltd.) and phosphatase inhibitors (cat. no. CW2383; Beijing Cowinbiocience Co., Ltd.). Cytoplasmic and nuclear extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (cat. no. 78833; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The protein concentration in each sample was determined using the BCA Protein Assay kit (cat. no. CW0014; Beijing Cowinbiocience Co., Ltd.) with bovine serum albumin as a standard. Equal amounts of protein (100 µg) lysate per sample were denatured in 5X Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) loading buffer (cat. no. CW0027; Beijing Cowinbiocience Co., Ltd.). Denatured proteins were separated on an 8-12% resolving gel and transferred onto nitrocellulose membranes (Pall Life Sciences) using a semidry transfer apparatus (Beijing Liuyi Biotechnology co., Ltd.). After being blocked with 5% bovine serum albumin (cat. no. 0332-100G; Amresco Inc.) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature, the membranes were probed with primary antibodies with gentle agitation overnight at 4˚C. After washing with TBST buffer, the membranes were incubated with appropriate HRP-conjugated secondary antibodies at room temperature for 1 h. After washing three times with TBST buffer, immunolabeled bands were detected by enhanced chemiluminescence. The integrated optical density (IOD) of the analyzed bands on the film was quantified using ImageJ software (National Institutes of Health; version 1.46). GAPDH and histone H3 served as cytoplasmic and nuclear internal controls, respectively. The levels of analyzed proteins were normalized to those of the internal control.

Semi-quantitative RT-PCR. Total RNA was isolated using the TRNzol method (cat. no. DP421; Tiangen Biotech Co., Ltd.). RNA (2 µg) was reverse transcribed with TransScript® First-Strand cDNA Synthesis SuperMix (cat. no. AT301; TransGen Biotech Co., Ltd.). The forward and reverse primers used for PCR were as follows: Tumor necrosis factor (TNF) -α forward, 5'-CGTAGCCACGCTGTAGAGAAA CCA-3' and reverse, 5'-CGCCAGTCGCTCAGAAAGCAG A AT-3'; XBP-1(u) forward, 5'-GTCGGAGACGGAACTGTTG TATTG-3' and reverse, 5'-GTCCTCTGGTGGTAGACCTCTG GGAG-3'; XBPl(s) forward, 5'-CTGAGTCCCGACGAGT GTCGC-3' and reverse, 5'-CAGGGTCCACCTTGGTCAGAA TG-3'; GAPDH forward, 5'-TGGCTGAGATGTGGTGAGGAG-3' and reverse, 5'-GTCTTCTGAGTGCGAGT3'. The primers were purchased from Sangon Biotech Co., Ltd. The PCR reaction conditions were as follows: 94˚C, 3 min; (4˚C, 30 sec; 55˚C, 30 sec; 72˚C, 1 min)x30 cycles; 72˚C, 5 min. The amplified products were separated on 1.5% agarose gels mixed with GoodView™ nucleic acid dye (cat. no. GV-2; Beijing SBS Genetech Co., Ltd.). Following electrophoresis, the agarose gel was visualized on a UV transilluminator and photographed. The IODs of the bands observed on the image were quantified using ImageJ software (National Institutes of Health; version 1.46). The transcription levels of the analyzed genes were normalized to those of GAPDH.

Statistical analysis. Data are expressed as the mean ± SD. Statistical analyses of the data were carried out by one-way ANOVA, followed by post hoc Tukey's tests. A value of P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using SPSS 19.0 software (IBM, Inc.).

Results

Overexpression of SERCA2a attenuates the upregulation of nuclear NF-κB and AP-1 DNA-binding activities following treatment of NRCMs with TM. At MOIs of 2, 4 and 10 pfu/cell, the expression level of SERCA2a was increased by 160, 110 and 90%, respectively, compared with that of the control group (Fig. 1). At an MOI of 2 pfu/cell, the viability of the NRCMs was 97.8%, similar to that of the control group. Unless otherwise stated, 2 pfu/cell was used as the preferred MOI in the subsequent experiments. When the MOI was >2 pfu/cell, the expression of SERCA2a was decreased, suggesting that the overexpression of SERCA2a may be cytotoxic. Following treatment with TM for 24 h, the DNA-binding activity of NF-κB in the TM group was increased by 4.1-fold (P<0.01; Fig. 2).
compared with the TM + rAd-EGFP and TM groups, the DNA-binding activity of NF-κB in the TM + rAd-SERCA2a group was decreased by 43.6% (P<0.01) and 66.0% (P<0.01), respectively. Following treatment with TM for 24 h, the DNA-binding activity of AP-1 in the TM group was increased by 26.9-fold (P<0.01). Compared with the TM + rAd-EGFP and TM groups, the DNA-binding activity of AP-1 in the TM + rAd-SERCA2a group was decreased by 60.2% (P<0.01) and 26.3% (P<0.01), respectively.

Overexpression of SERCA2a at an MOI of 1 pfu/cell still attenuates the upregulation of nuclear NF-κB and AP-1 DNA-binding activities following treatment of NRCMs with TM. As previously demonstrated, under limited exposure to calf serum, compared with the non-infected control group, the size, protein content and protein synthesis rate in the infected rat myocytes exhibited a more rapid increase (32). Tauroursodeoxycholic acid (TUDCA), a recognized ERS inhibitor, was used as a control in this experiment. The
synchronization time was delayed to that prior to the addition of TM (final concentration, 10 µg/ml), instead of that prior to infection. Considering that NRMs are prone to the ER overload response (EOR) induced by rAd-SERCA2a infection, the MOI was reduced to 1.0 pfu/cell. Compared with the TM + rAd-EGFP and TM groups, the overexpression of SERCA2a significantly attenuated the upregulation of NF-κB (both P<0.05) and the AP-1 DNA-binding activities (both P<0.05), respectively. The results were similar to those observed at 2 pfu/cell (Fig. 3). The results of EMSA revealed that TUDCA significantly attenuated the upregulation of NF-κB and AP-1 DNA-binding activities induced by TM, corroborating the successful construction of the cellular model of ERS. The supershift assays revealed that activated NF-κB in the nucleus contained p50 and p65 subunits, and activated AP-1 in the nucleus contained c-Jun and c-Fos subunits (Fig. 3).

Overexpression of SERCA2a attenuates the upregulation of nuclear NF-κB and AP-1 DNA-binding activities induced by H/R. In the H/R model, the overexpression of SERCA2a significantly attenuated the upregulation of NF-κB and AP-1 DNA-binding activities (Fig. 4), similar to the findings observed with the TM model.

Overexpression of SERCA2a attenuates the activation of the IRE1α signaling pathway induced by TM in the NRMs. Compared with the vehicle control group, the protein levels of phospho-PERK (Thr980) (Fig. 5), phospho-IRE1 (Ser724) (Fig. 6), BiP, CHOP and cleaved caspase-12 (Fig. 7) in the TM group were significantly increased. No significant decreases were observed in the phospho-PERK (Thr980) and phospho-eIF2α (Ser51) levels in the TM + rAd-SERCA2a group compared with the TM + rAd-EGFP group (Fig. 5). Compared with the TM + rAd-EGFP and TM groups, the ratio of phospho-IRE1 to unphosphorylated IRE1 in the TM + rAd-SERCA2a (MOI=2.0) group was reduced by 25% (P<0.01) and 31.8% (P<0.01), respectively (Fig. 6). The results of semi-quantitative RT-PCR revealed that compared with the TM + rAd-EGFP and TM groups, the ratio of spliced active XBP1 to unspliced inactive XBP1 in the TM + rAd-SERCA2a (MOI=2.0) group was reduced by 20.5% (P<0.01) and 20% (P<0.05), respectively.
Overexpression of SERCA2a attenuates ERS-associated apoptosis. BiP, also known as Grp78, is one of the molecular markers of ERS. The overexpression of SERCA2a decreased the expression of BiP, compared with that in the TM + rAd-EGFP group. CHOP (also known as GADD153) and caspase-12 are relevant to ERS-associated apoptosis. Compared with the TM + rAd-EGFP group, the expression of CHOP and the ratio of cleaved caspase-12 to pro-caspase-12 in the TM + rAd-SERCA2a group were decreased by 40% (P<0.05) and 56% (P<0.01), respectively (Fig. 7). Compared with the TM group, the expression of CHOP and the ratio of cleaved caspase-12 to pro-caspase-12 were decreased by 23% (P>0.05) and 3.9% (P>0.05), respectively. These findings indicated that the overexpression of SERCA2a attenuated ERS-associated apoptosis.

Overexpression of SERCA2a induces EOR. The overexpression of molecules resident in the ER can lead to EOR. EOR is characterized by NF-κB activation. In the TM + rAd-SERCA2a group, the nuclear translocation of NF-κB was significantly increased by 1.40-fold (P<0.05), the transcription level of TNF-α increased by 87.4% (P<0.05)
Figure 5. Effects of the overexpression of SERCA2a on the activation of the PERK signaling pathway following treatment with TM for 24 h. (A) Western blot bands corresponding to phospho-PERK, unphosphorylated PERK, phospho-eIF2α, and eIF2α. GAPDH was used as an internal control. (B) Bar graph showing the ratio of the IODs of bands corresponding to phospho-PERK from (A) to that of unphosphorylated PERK. (C) Bar graph showing the ratio of the IODs of bands corresponding to phospho-eIF2α from (A) to that of eIF2α. Data are representative of three independent experiments (mean ± SD). P<0.05 vs. TM; P<0.01 vs. control. SERCA2a, sarco/endoplasmic reticulum Ca2+-ATPase; PERK, double-stranded RNA-dependent protein kinase (PKR)-like ER kinase; TM, tunicamycin; eIF2α, eukaryotic protein synthesis initiation factor 2; IOD, integrated optical density.

Figure 6. Effects of the overexpression of SERCA2a on the activation of the IRE1α signaling pathway following treatment with TM. (A) Western blot bands corresponding to phospho-IRE1 and unphosphorylated IRE1. (B) Bar graph showing the ratio of the IODs of bands corresponding to phospho-IRE1 from (A) to that of unphosphorylated IRE1. (C) RT-PCR products corresponding to XBP1(s) and XBP1(u) following agarose gel electrophoresis. (D) Bar graph showing the ratio of the IODs of bands corresponding to XBP1(s) from (C) to that of XBP1(u). Data are representative of three independent experiments (mean ± SD). P<0.05 vs. TM + rAd-EGFP; P<0.05 vs. TM; P<0.01 vs. Control. SERCA2a, sarco/endoplasmic reticulum Ca2+-ATPase; IRE1α, inositol-requiring 1α; TM, tunicamycin; IOD, integrated optical density; XBP1, X-box binding protein-1; EGFP, enhanced green fluorescent protein.
Overexpression of SERCA2a exhibited an increasing trend (P>0.05) compared with the TM + rAd-EGFP group, which suggested that the overexpression of SERCA2a induced EOR (Fig. 8).

**Overexpression of SERCA2a decreases the level of nuclear phospho-p65 (Ser536).** The increase in the NF-κB p65 nuclear translocation and the attenuation of the upregulation of NF-κB p65 DNA-binding activity due to the overexpression of SERCA2a appeared paradoxical. To address this issue, the effects of overexpression of SERCA2a on post-translational modifications of NF-κB p65 were further explored. Compared with that in the TM + rAd-EGFP group, the ratio of nuclear phospho-NF-κB p65 (ser536) to NF-κB p65 in the TM + Ad-SERCA2a group was significantly decreased by 59.6% (P<0.05; Fig. 9).

**Discussion**

H9c2 cells lack NF-κB p50 expression (33); therefore, this cell line was not selected as the study object. TM blocks N-linked glycosylation and is widely used to induce UPR. In this cell-based study, ERS-associated inflammation was induced by TM, thus preventing interference from tissue and circulating immune cells.

The present study demonstrated that TM induced a significant increase in the NF-κB DNA-binding activity and in the nuclear translocation of NF-κB. The addition of the ERS protectant, TUDCA, prior to treatment with TM significantly attenuated the upregulation of DNA-binding activity of NF-κB and AP-1. These findings indicate that the cellular TM-induced ERS model was successfully constructed.

Hamid *et al* (33) revealed that in HF, persistent activation of NF-κB p65 in myocytes aggravates ventricular remodeling by conferring pro-inflammatory, profibrotic and pro-apoptotic effects. It appears important to control the activation of NF-κB in HF. The UPR and NF-κB are interconnected through various mechanisms. In the present study, it was found that the overexpression of SERCA2a attenuated ERS and the activation of the IRE1α signaling pathway in the NRcMs induced by TM, resulting in the attenuation of the upregulation of NF-κB and AP-1 DNA-binding activities.

The accumulation of wild-type or misfolded proteins in the ER results in the release of Ca²⁺ from the ER. This leads to the generation of ROS, activating NF-κB. This process is called the EOR (34). Some viral proteins, such as the virion surface hemagglutinin (35), C-terminal truncation of the middle surface antigen from hepatitis B virus (36), adenovirus E3/19K protein (37) and human hepatitis C virus NS5A protein (38),...
can cause the EOR. The overexpression of SERCA2a in COS cells increases the calcium uptake rate; however, the overexpression of SERCA2a also induces cellular calcium overload and death (39). O’Donnell et al (32) proposed that in neonatal cardiomyocytes, the SR system was not well developed, and the SR volume was limited. A several-fold increase in SERCA within 2- to 3-day period can induce the dense accumulation of SERCA molecules in the limited SR space and leads to the disorder of membrane structure and function, resulting in perturbation of calcium homeostasis (32). These earlier findings indicate that exogenous expression of SERCA can cause EOR, although NF-κB activation and TNF-α transcription have not been investigated. The window of MOIs between exogenous gene expression and production of cellular toxicity is narrower for the overexpression of SERCA than for EGFP. Wu et al found that at an MOI of 4 pfu/cell, the overexpression of SERCA1 induced the loss of NRCMs and DNA fragmentation (40). O’Donnell et al (32) suggested that the optimal MOI of adenoviral vector carrying wild-type SERCA1 is in the range of 2 to 4 pfu/cell in NRCMs. This titer increased SERCA activity by >2-fold and enhanced the kinetics of Ca²⁺ transients.

The present study identified 2 pfu/cell as the preferred MOI based on the expression level of exogenous SERCA2a, cell viability and LDH leakage, thus minimizing the cytopathic effects. However, at this MOI, the detachment of cells can still be observed under an inverted microscope. It was found that the nuclear translocation of NF-κB p65 in the TM + rAd-SERCA2a group was significantly increased following treatment with TM compared with that in the TM + rAd-EGFP group, which was consistent with the occurrence of the EOR.

However, the mechanisms through which the accumulation of proteins in the ER membrane increase Ca²⁺ permeability remain unclear. Pahl (34) proposed that the accumulation of membrane proteins may impair SERCA function, or the Ca²⁺ permeability of the ER membrane may be aggravated due to
Studies have demonstrated that the overexpression of SERcA2a can enhance its pump function, the latter possibility is more reasonable in the case of overexpression of SERcA2a-induced NF-κB translocation and the attenuation of the upregulation of NF-κB-induced transcription in the group overexpressing SERCA2a (data not shown); however, further repeated experiments are required to confirm this conclusion. It was hypothesized that the preceding EOR induced by accumulation of exogenous SERCA2a in sarcoplasmic reticulum might precondition the cells against subsequent TM-induced upregulation of NF-κB and AP-1 DNA-binding activities. Further studies to investigate C/EBP-β-p65 complexes and TRAF2 are required to substantiate this view.

It remains unclear as to whether EOR induced by SERCA2a overexpression was involved in alleviating ERS-related apoptosis in the present study. As it is well known that the increased SERCA2a expression can maintain calcium homeostasis and attenuate ERS, it could not be determined whether EOR can precondition the NRCSMs against subsequent ERS-induced apoptosis. It is best to include another group to block NF-κB and/or TNFα receptor signaling pathway to test this hypothesis.

Wu et al (40) revealed that the effects of adenoviral vector carrying SERCA1 on NRCSMs and adult rat cardiomyocytes (ARCMs) were differed significantly. The infection of NRCSMs at an MOI of 4 pfu/cell led to apoptosis. At an optimal MOI,

Figure 9. Effects of the overexpression of SERCA2a on the nuclear level of phospho-NF-κB p65 (ser536) in NRCSMs following treatment with TM for 24 h. (A) Western blot bands corresponding to nuclear phospho-NF-κB p65 (ser536). Arrow indicates the position of phospho-NF-κB p65 (ser536) band. (B) Bar graph showing the ratio of theIODs of bands corresponding to the nuclear phospho-NF-κB p65 (ser536) from (A) to that of NF-κB p65. Three independent experiments were performed (mean ± SD). *P<0.01 vs. TM; #P<0.01 vs. TM + rAd-EGFP. P<0.01 vs. TM. SERCA2a, sarco/endoplasmic reticulum Ca2+-ATPase; NRCMs, neonatal rat cardiomyocytes; TM, tunicamycin; IOD, integrated optical density.
the protein level of SERCA1 in NRCMs was 4-fold higher than that in the ARCMs, and the activity of Ca\(^{2+}\)-ATPase increased by 4-fold in the NRCMs, but only by 1.5-fold in the ARCMs. It should be pointed out that since adenoviral vector carrying SERCA1 has no apoptotic effect on ARCMs (40), the findings of the present study using NRCMs cannot be extrapolated to explain the results of AA V1-SERCA2a gene therapy in the CUPID 2 study. In a previous rat pressure overload HF model, the intracoronary delivery of adenoviral vector carrying SERCA2a induced reductions in the serum levels of interleukin (IL)-1, IL-6 and TNF-α; however, local inflammation of the heart was not investigated (47). To prevent the interference from EOR, it is better to undertake similar experiments in ARCMs.

There are some limitations associated with the present study. At the beginning of the experiment, it was not expected that the EOR would have such a profound impact on the experimental results. After obtaining the results, it was determined that the overexpression of SERCA2a leads to EOR, which would greatly interfere with the study of ERS-related inflammation. The authors thus aim to perform further research on ARCMs in the future. As shown in Fig. 9B, compared with the other three groups, the total p65 content in the nuclear compartment of untreated cardiomyocytes was minimal. When calculating the ratio of phosphorylated p65 to p65 in the control group, the ratio may become unreliable. IL-1β, IL-6 and MCP-1 were detected in the culture medium supernatant in the present study; however, since these experiments were not repeated a sufficient number of times, the data were not presented. It is preferable to use more sensitive methods, such as reporter gene plasmid transfection to confirm the conclusions. In addition to caspase-12, it is preferable to evaluate more indicators related to apoptosis, such as caspase-3, poly(ADP-ribose) polymerase and Annexin V, in order to strengthen these conclusions.

In conclusion, in the cellular TM-induced ERS-associated inflammation model, the overexpression of SERCA2a in the NRCMs induced EOR, approximately two days prior to TM-induced UPR. The results suggested that the overexpression of SERCA2a had a ‘double-edged sword’ effect on ERS-associated inflammation. On the one hand, the overexpression of SERCA2a attenuated ERS and the activation of IRE1α signaling pathway induced by TM, resulting in the attenuation of the upregulation of NF-κB and AP-1 DNA-binding activities. However, on the other hand, the overexpression of SERCA2a induced EOR, leading to the further nuclear translocation of NF-κB and the transcription of TNF-α. The preceding EOR may precondition the NRCMs against subsequent ERS-associated inflammation induced by TM. The findings of the present study may enhance the current understanding of the pros and cons of the overexpression of SERCA2a in the NRCMs and inspire the further exploration of the underlying mechanisms of the preconditioning effects induced by the EOR. Elucidating the aforementioned mechanisms may help to identify novel treatments for heart diseases in the future. Further studies performed using ARCMs are required to prevent the interference of the EOR, in which SERCA2a overexpression can be achieved through AA V1-SERCA2a transfection or constructing transgenic animal models.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
XLu and XLi were involved in the conception of the study, applying for funds and revising the manuscript. ZQ, YQ and TT performed the experiments. ZQ prepared the draft of the manuscript. XLiu was involved in designing part of the study and revising the manuscript. ZQ and X Lu confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011) and the animal experimentation guidelines of the Chinese PLA General Hospital.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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