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Calumenin has a role in the alleviation of ER stress in neonatal rat cardiomyocytes

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ABSTRACT

Disturbance of endoplasmic reticulum (ER) homeostasis causes ER stress (ERS), and triggers the unfolded protein response (UPR) that consequently reduces accumulation of unfolded proteins by increasing the quantity of ER chaperones. Calumenin, a Ca^{2+}-binding protein with multiple EF hand motifs, which is located in the ER/SR, is highly expressed during the early developmental stage of the heart, similar to other ER-resident chaperones. The aim of this study was to investigate the functional role of calumenin during ERS in the heart. Like other chaperones (e.g., GRP94 and GRP78), calumenin expression was highly upregulated during ERS induced by 10 μg/ml tunicamycin, but attenuated in the presence of 500 μM PBA, the chemical chaperone in neonatal rat ventricular cardiomyocytes (NRVCs). Upon 7.5-fold overexpression of calumenin using a recombinant adenovirus system, the expression levels of ERS markers (GRP78, p-PERK, and p-eIF2α) and ER-initiated apoptosis markers (CHOP and p-JNK) were reduced, whereas the survival protein BCL-2 was upregulated during ERS compared to the control. Evaluation of cell viability by TUNEL assay showed that apoptosis was also significantly reduced by calumenin overexpression in ERS-induced cells. Taken together, our results suggest that calumenin plays an essential role in the alleviation of ERS in neonatal rat cardiomyocytes.

1. Introduction

The endoplasmic reticulum (ER) is an intracellular organelle involved in multiple cellular processes such as protein synthesis, lipid biosynthesis, protein folding, posttranslational modification, transport of proteins, and regulation of calcium homeostasis. Perturbation of ER function, which is caused by various factors, such as ischemia, hypoxia, exposure to free radicals, elevated protein synthesis, and gene mutation, can lead to accumulation of unfolded and misfolded proteins in the ER, a condition referred to as ER stress (ERS) [1–3]. ERS induces the unfolded protein response (UPR), an adaptive response to restore ER homeostasis by reducing the accumulation of unfolded proteins. When ERS occurs, the ER molecular chaperone Bip/GRP78 dissociates from the three ER trans-membrane sensors—protein kinase-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6)—leading to their activation. Activation of UPR results in translational inhibition, thereby reducing the production of new proteins in ER, transcriptional activation of genes for chaperones and folding enzyme, and promotion of ER-associated degradation (ERAD) to remove misfolded proteins. In the heart, ER chaperones such as GRP94, GRP78, and calreticulin are upregulated in response to UPR to enhance the ability of the SR to regulate its intracellular Ca^{2+} level and to reduce the quantity of unfolded proteins by supporting their proper folding, a cardioprotective effect [4–7].

Nevertheless, if the stress is severe or prolonged, the UPR fails to protect cells against ERS. The signaling switches from pro-survival (adaptive response) to pro-apoptosis (maladaptive response) by transcriptional induction of C/EBP homologous protein (CHOP) or by the activation of c-Jun N-terminal kinase (JNK) and caspase-12–dependent pathways [8,9]. Recently, the UPR and ER-initiated apoptosis have been implicated in the progression of cardiovascular diseases such as ischemic and non-ischemic heart failures [4,7,10–12]. Therefore, pathways for coping with ERS may be an important target for the development of therapeutic approaches against cardiovascular diseases.

Abbreviations: ATF, activating transcription factor; Ad-calu, adenovirus containing mouse calumenin 1 cDNA; Ad-lacZ, adenovirus containing lacZ gene; CHOP, C/EBP-homologous protein; ERAD, ER-associated degradation; eIF2, eukaryotic initiation factor 2; ERp57, the thiol oxidoreductases proteins disulphide isomerase and ER protein; GRP, glucose related protein; ifu, infectious unit; IRE, inositol-requiring enzyme; MOI, multiplicity of infection; NRVCs, neonatal rat ventricular cardiomyocytes; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PBA, 4-phenylbutyric acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tg, thapsigargin; TM, tunicamycin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UPR, unfolded protein response.

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Calumenin is a Ca^{2+}-binding protein located in the SR lumen of mammalian heart and belongs to the CREC family of Ca^{2+}-binding proteins having multiple EF-hand domains [13]. Calumenin is ubiquitously expressed, albeit at a higher expression level in the heart. It has also been reported that the mRNA expression level of calumenin in embryonic heart is higher than that in adult mouse heart [14]. Likewise, calumenin protein expression markedly decreases and achieves a steady state in adult mouse heart compared to embryonic and neonatal heart [15]. Similarly, the expression pattern of several ER resident chaperones such as calreticulin, GRP78, GRP94, protein disulfide isomerase (PDI) and ER protein 57 (ERp57) were found to be highly expressed during earlier stages of development [16]. It has been observed that overexpression of ER-resident molecular chaperones, such as GRP78 and GRP94, can protect cells from cell death by attenuating ERS [17–19]. Further studies have shown that chemical chaperones can attenuate the progression of cardiovascular diseases [10,20]. Thus, this study is interested in exploring whether calumenin, a multifunctional CREC family protein, has a chaperone-like role in the heart.

In the present study, we explored the possible chaperone-like role of calumenin for ERS and ER-initiated apoptosis using the recombinant adenovirus overexpression system in neonatal rat ventricular cardiomyocytes (NRVCs). The results showed that overexpression of calumenin significantly alleviated ERS and inhibited ER-initiated cellular apoptosis, suggesting that calumenin may be an important molecular chaperone in NRVCs.

2. Materials and methods

2.1. Preparation of neonatal rat ventricular cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated from 2-day-old neonatal Sprague–Dawley rats using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, NJ, USA). The aortic root and atria were removed prior to isolation. Isolated cells were plated in gelatin-coated culture dishes. The number of cells was approximately 1×10^6 cells per 60-mm dish. Cells were suspended in DMEM (low glucose) with 10% fetal bovine serum (FBS), 1% antibiotics, and 0.1 mM BrdU, followed by incubation with 5% CO_2 and 98% relative humidity at 37 °C in a culture incubator. All animal procedures were approved by the GIST Institutional Animal Care and Use Committee.

2.2. Recombinant adenovirus amplification

Previously generated recombinant adenovirus containing mouse calumenin-1 cDNA (Ad-calu) [21] was amplified in HEK 293 cells. Adenovirus containing the lacZ gene (Ad-lacZ) was used as a control. The infectious unit (ifu) of amplified viruses was determined by titration using the Adeno-X rapid titer kit purchased from Clontech Laboratories (Mountain View, CA, USA). Various multiplicities of infection (MOIs) of viruses were used for generating calumenin overexpression in NRVCs.

2.3. Adenoviral infection of NRVCs and treatment of the infected cells with reagents to trigger ERS

After a 24 h incubation period of NRVCs in the low glucose DMEM media (as shown in Section 2.1), the media were replaced with a serum (FBS)-free media to starve the cells. During the starvation period, the recombinant adenoviruses were infected for 6 h followed by a 1× change into serum-free media. After an additional 12 h maintenance period, 10 μg/ml TM or 2 μM Tg was treated for 12 h to induce ERS. To observe the effect of a chemical chaperone, 500 μM PBA was treated in the presence or absence of 10 μg/ml TM and incubated for 24 h.

2.4. Western blot analysis

To check the expression levels of proteins, Western blot analysis was performed. Cells were lysed in 100 μL SDS lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris–HCl with protease inhibitor cocktail). The BCA protein assay kit (Pierce, Rockford, IL, USA) was used for protein quantification. Cardiomyocyte lysate was solubilized in 5× sample buffer containing β-mercaptoethanol, and the constituent proteins were separated by SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were then incubated with 5% skim milk or 5% BSA in Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 (TBST) for 1 h at room temperature to block non-specific binding of antibody. Next, the membranes were incubated overnight at 4 °C with primary antibodies against one of the following proteins: calumenin, KDEL (GRP78, 94), p-PERK, PERK, p-eIF2α, CHOP, Bcl-2, p-JNK, JNK, α-tubulin, and GAPDH. After primary antibody incubation, membranes were washed with TBST and further incubated with the appropriate peroxidase-conjugated secondary antibody. Western blot band signals were detected by using the Image Quant Las 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and SuperSignal West Pico Chemiluminescence Kit (Thermo Fisher Scientific, Inc., Waltham, MA). Band intensities were measured by ImageJ software (NIH).

2.5. TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed to detect and quantify apoptotic cell death at the single cell level. NRVCs were plated on gelatin-coated cover slips, incubated and infected by recombinant adenoviruses at MOI 50, and treated with 10 μg/ml TM. After 12 h of TM treatment, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. The slides were washed with PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Nuclear staining was accomplished by Hoechst 33342, and apoptotic cells were labeled by a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase using In Situ Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany). After incubation for 1 h at 37 °C, the stained slides were washed 3 times with PBS and observed under a Olympus confocal microscope FV1000 (Olympus, Tokyo, Japan). The number of TUNEL-positive cells was measured using Metamorph software.

2.6. Statistical analysis

The experimental values are represented as mean ± S.E.M. Multiple comparisons between the different types of samples were conducted using an ANOVA test. A value of $P < 0.05$ was used as the criterion for identifying statistically significant differences.

3. Results

3.1. Calumenin was upregulated by ER stress in NRVCs

In order to investigate whether calumenin is involved in the alleviation of ERS in NRVCs, the cells were treated with 10 μg/ml tunicamycin (TM) and the expression levels of calumenin were examined. Calumenin was upregulated by ERS in a manner similar to GRP78, the marker protein of ERS (Fig. 1). The downward shift in molecular weight of calumenin after addition of TM may be due to the deglycosylation of calumenin, since TM blocks the synthesis of

all N-linked glycoproteins. We also examined whether the chemical chaperone 4-phenylbutyric acid (PBA) [13] affects the protein level of calumenin during ERS. The result revealed that the significantly increased expression of calumenin due to TM induction was abrogated in the presence of 500 μM PBA, suggesting that PBA blocks TM-mediated UPR. The increased GRP78 expression observed by TM induction was also significantly ($P < 0.05$) inhibited by 500 μM PBA.

3.2. Adenovirus-mediated overexpression of calumenin alleviated ERS in NRVCs

In the present study, the adenovirus gene transfer system was adopted for calumenin overexpression in NRVCs to investigate the functional role of calumenin during ERS responses. Cultured cardiomyocytes were infected with adenovirus encoding calumenin (Ad-calu), with Ad-lacZ serving as a control. The calumenin expression level was examined by Western blotting according to multiplicity of infection (MOI) to determine the optimal protein expression 30 h after infection. Calumenin expression increased 7.5-fold at MOI 10, 13.9-fold at MOI 50, and 17.9-fold at MOI 100 (Supplementary Fig. S1A and B). Overexpression of calumenin alone did not cause ERS, as indicated by GRP94/78 protein levels (Supplementary Fig. S1A and B). The condition of MOI 10 was selected for subsequent studies to eliminate any possible non-specific effect of overexpressed calumenin (see Supplementary Fig. S1).

Ad-calu–infected cells were treated for 12 h with 10 μg/ml TM or 2 μM thapsigargin (Tg), an ERS inducer through ER calcium depletion. The expression levels of GRP78 (the ERS marker protein), phosphorylated PERK (p-PERK; the UPR signaling pathway protein), and the downstream phosphorylated eukaryotic translation initiation factor 2α (p-eIF2α) were all examined by Western blotting. The increased expression level of GRP78 induced by either TM or Tg was significantly decreased in Ad-calu–infected cells compared with Ad-lacZ (Fig. 2 and Supplementary Fig. S2). Furthermore, the levels of p-PERK and p-eIF2α were also decreased by Ad-calu infection in TM- or Tg-treated cells. Taken together, the results indicate that ERS is attenuated by overexpression of calumenin, suggesting that calumenin is involved in the alleviation of ERS in NRVCs, similar to that observed in other molecular chaperone proteins.

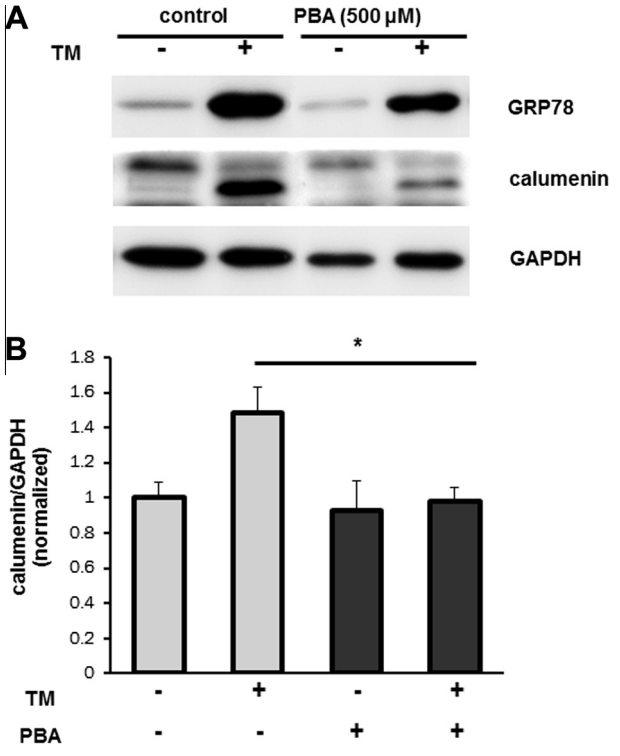

Fig. 1. Calumenin was upregulated by ER stress in NRVCs. (A) According to the Western blot data, the expression levels of GRP78 and calumenin were markedly increased in response to 10 μg/ml TM-induced ERS for 24 h in NRVCs. However, the increased expression levels of both proteins by ERS were downregulated in the presence 500 μM PBA. Note that the molecular weight of calumenin was downshifted in the presence of TM due to the deglycosylation (see text). (B) Summary relative expression levels of calumenin during ERS induced by treatment with 10 μg/ml TM for 24 h in the presence or absence of 500 μM PBA in NRVCs. Data represent mean ± S.E.M. $^*P < 0.05$, $n = 3$.

3.3. Adenovirus-mediated overexpression of calumenin reduced ERS-associated apoptosis in NRVCs

It has been well established that C/EBP homologous protein (CHOP) is a pro-apoptotic transcription factor, which can mediate transcriptional induction of BIM, a pro-apoptotic BH3-only protein, while inhibiting Bcl-2, an anti-apoptotic protein, during ER-initiated apoptosis (see Fig. 4B). Phosphorylated JNK and active caspase-3 are also actively involved in this process. Accordingly, severe or prolonged ERS could lead to ER-initiated apoptosis in cardiomyocytes, which could eventually lead to heart failure [3,9,12]. In order to investigate the effect of calumenin overexpression on ER-initiated apoptosis, immunoblotting experiments were performed using NRVCs. Upon addition of either 10 μg/ml TM or 2 μM Tg, the expression levels of CHOP and p-JNK were significantly increased, while during calumenin overexpression, the ERS-induced upregulation of CHOP and p-JNK was suppressed (Fig. 3 and Supplemental Fig. S3). Moreover, the reduced expression level of the anti-apoptotic protein Bcl-2 by ERS was increased in Ad-calu infected cells (Fig. 3C and Supplemental Fig. S3C). These results suggest that overexpression of calumenin prevents cardiomyocytes from undergoing ER-initiated apoptosis.

To examine whether the overexpression of calumenin also affects cell survival by attenuating ER-initiated apoptosis, terminal deoxynucleotidyltransferase-mediated dUTP Nick-End Labeling (TUNEL) assay was performed. TUNEL assay results revealed a reduction in the number of TUNEL-positive cells by 15.5% in Ad-calu–infected cells when compared with Ad-lacZ, suggesting that overexpression of calumenin reduced apoptosis in cardiomyocytes (Fig. 4). This finding indicates that overexpression of calumenin has a survival effect on ER-initiated apoptotic cells.

4. Discussion

Calumenin is a multiple EF-hand Ca^{2+}-binding protein that belongs to the CREC family and possesses diverse functions in different tissues [13,14]. Evidence has shown that abnormal expression of calumenin is associated with multiple pathological conditions such as cardiomyopathy and colon cancers [22,23]. However, the etiology of the diseases remained to be elucidated. The level of calumenin proteins steadily decreases in mouse heart until it reaches a steady-state level that is maintained throughout adulthood [13,14], indicating that the pattern of calumenin expression is similar to that of other SR/ER chaperone proteins (e.g., calreticulin, GRP78/94 and ERp57). However, the possible role of calumenin as a molecular chaperone in SR/ER has not been explored.

In the present study, we examined the possible chaperone-like role of calumenin during ERS responses by using a genetically manipulated calumenin overexpression system in NRVCs. The key findings of this study are as follows: (1) calumenin was significantly upregulated during ERS, an upregulation that was significantly diminished in the presence of PBA (Fig. 1); (2)

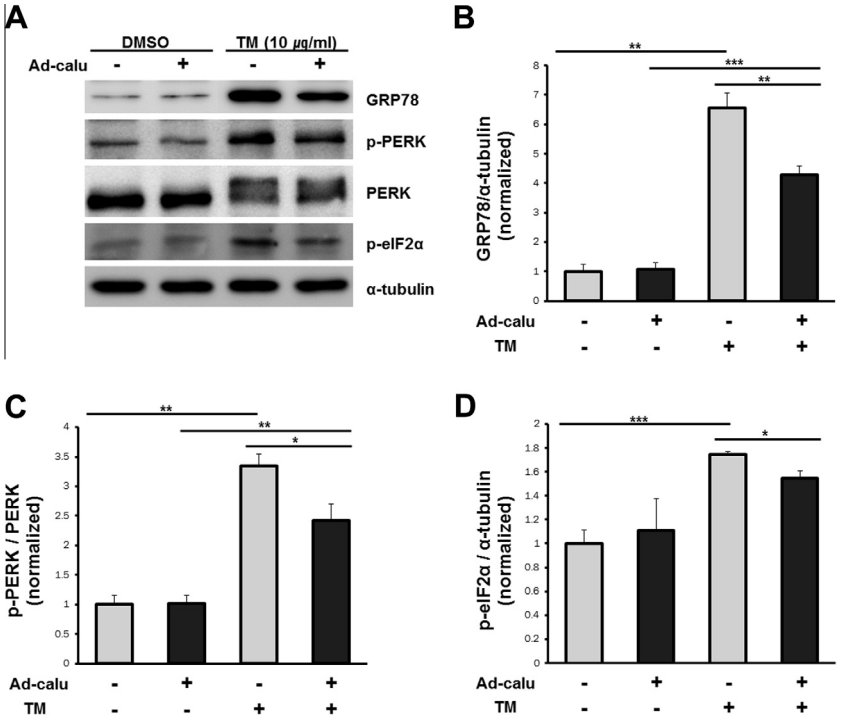

Fig. 2. Overexpression of calumenin alleviated ERS-mediated UPR in NRVCs. (A) Western blot analyses for ERS sensor (GRP78) and UPR pathway proteins (p-PERK and p-eIF2α) in NRVCs infected with Ad-lacZ or Ad-calu at MOI 10 (7.5X overexpression) for 18 h and further treated with 10 μg/ml TM for 12 h are shown. Summary relative expression levels of GRP78/ α-tubulin (B), p-PERK/PERK (C) and p-eIF2α/α-tubulin (D) in Ad-lacZ or in Ad-calu infected NRVCs with or without treatment of 10 μg/ml TM are shown. Band intensities of the tested proteins were normalized to those for the Ad-lacZ samples (ad-calu–/TM-). Data represent mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3–5$.

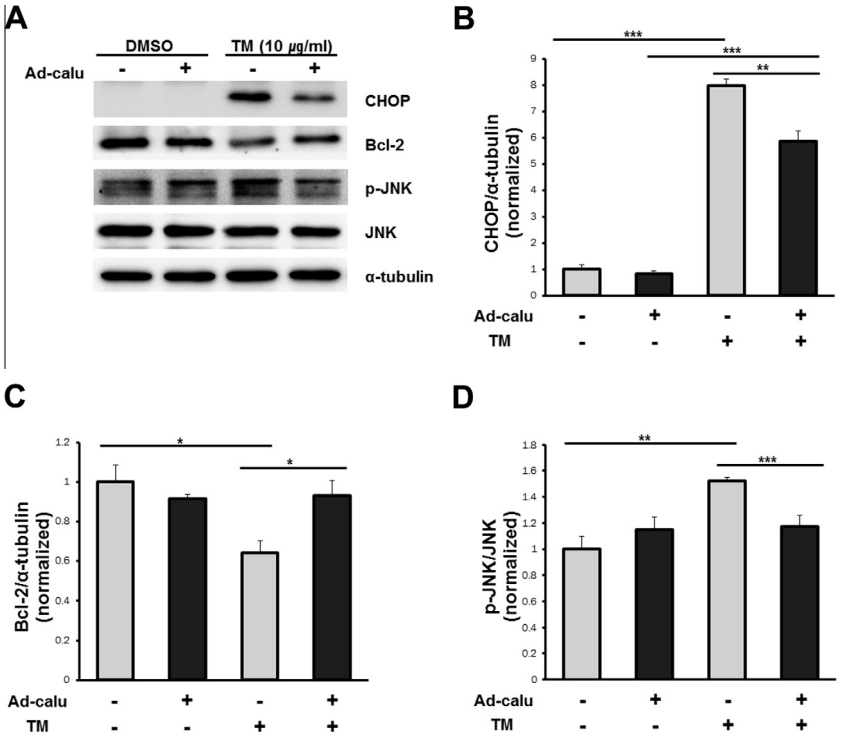

Fig. 3. Overexpression of calumenin alleviated ER-initiated apoptosis in NRVCs. (A) Western blot analyses of proteins involved in ER-initiated apoptosis signaling (CHOP, Bcl-2, p-JNK) in NRVCs infected with Ad-lacZ or Ad-calu at MOI 10 (7.5× overexpression) for 18 h and treated with 10 μg/ml TM for 12 h to trigger ERS are shown. Summary relative expression levels of CHOP/α-tubulin (B), Bcl-2/α-tubulin (C) and p-JNK/JNK (D) in Ad-lacZ or in Ad-calu infected NRVCs with or without treatment of 10 μg/ml TM are shown. Band intensities of the tested proteins were normalized to those for the Ad-lacZ samples (ad-calu–/TM-). Data represent mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3–5$.

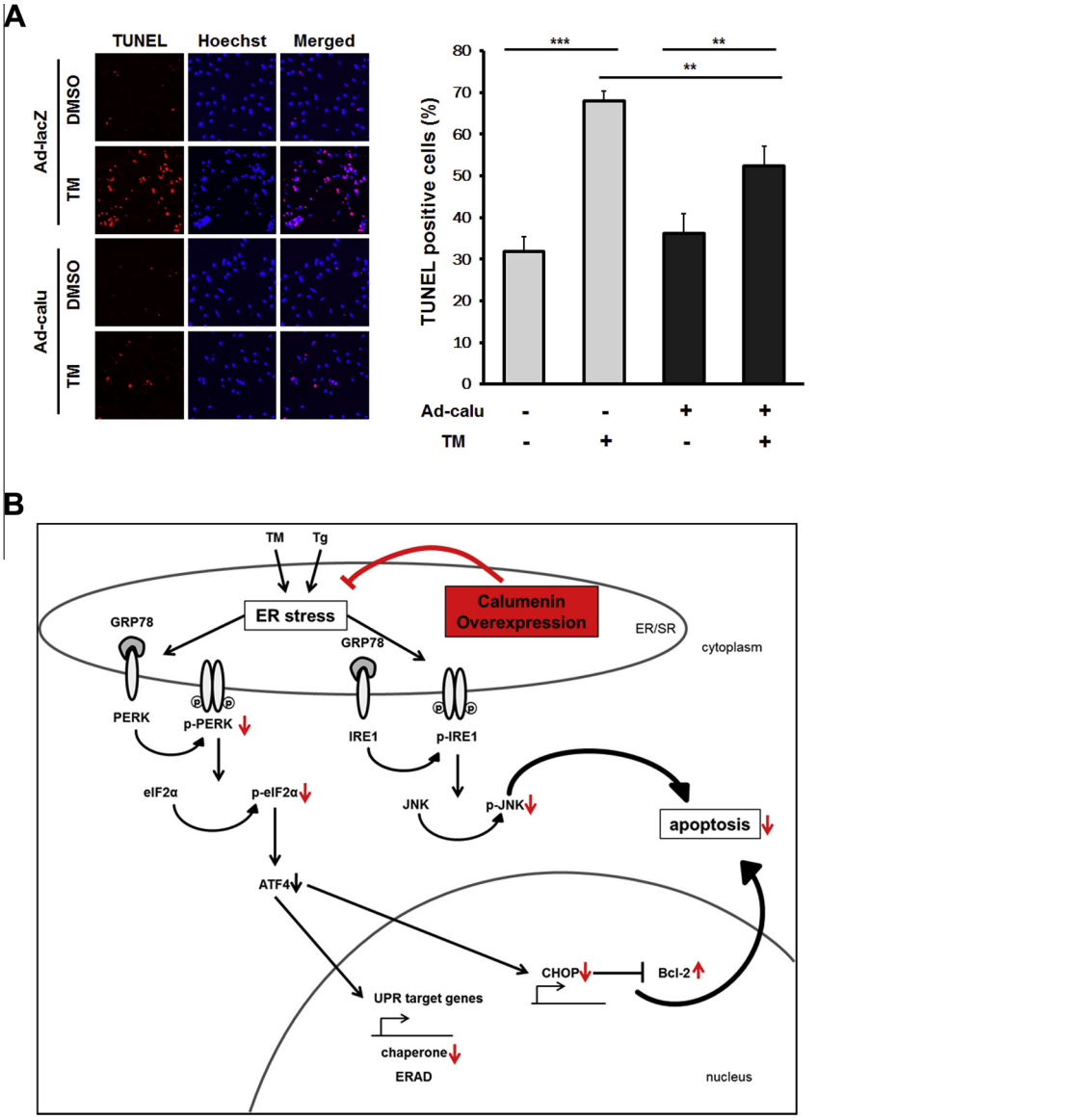

Fig. 4. Overexpression of calumenin enhanced cell survival during ERS and UPR in NRVCs. (A) The images of TUNEL assays for NRVCs treated with 10 μg/ml TM for 12 h after infection with Ad-lacZ or Ad-calu at MOI 50 for 18 h are shown. Nuclei of apoptotic cells (TUNEL-positive cells) are shown in red and nuclei of normal cells are shown in blue with Hoechst33342 staining. Numbers of the merged areas (pink) were measured for counting dead cells. Summary quantities of TUNEL-positive cells in response to the treatment of TM in Ad-calu infected NRVCs. Data represent mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 21–24$. (B) The schematic diagram shows attenuation of ERS and ER-initiated apoptosis by overexpression of calumenin in NRVCs. Tunicamycin (TM) or thapsigargin (Tg) induces ERS with phosphorylation and activation of PERK and eIF2α and up-regulation of GRP78. As a result of attenuating ERS by overexpression of calumenin, the expression levels of GRP78, p-PERK and p-eIF2α are reduced. Furthermore, prolonged or severe ERS induces ER-initiated apoptosis via CHOP dependent pathway and JNK pathway. Overexpression of calumenin alleviates apoptosis by reducing the expression levels of CHOP and phosphorylated JNK. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

overexpression of calumenin attenuated ERS as evidenced by the downregulation of GRP78, p-PERK, and p-eIF2α (Fig. 2 and Supplemental Fig. S2); (3) calumenin reduced ER-initiated apoptosis in NRVCs as evidenced by downregulation of CHOP and p-JNK and upregulation of Bcl2 (Fig. 3 and Supplemental Fig. S3); and (4) overexpression of calumenin has a survival effect on ER-initiated apoptotic cells (Fig. 4). Taken together, the present study suggests that calumenin plays a chaperone-like role in NRVCs through alleviation of ERS and downregulation of ER-initiated apoptosis as shown in Fig. 4B.

During cellular stresses such as oxygen deprivation, glucose starvation, inhibition of protein glycosylation, and ER Ca^{2+} depletion, the expression levels of ER chaperones are increased to prevent aggregation of proteins and to promote proper protein folding [4–7,24]. Several ER chaperones, such as GRP78, GRP94, calreticulin, and calnexin, are upregulated by accumulation of unfolded proteins in the ER [25,26]. The present study showed that calumenin was also upregulated during ERS. However, the upregulation was alleviated in the presence of PBA, a chemical chaperone (Fig. 1), suggesting that calumenin responds to ERS in a similar manner to other ER chaperones.

In the present study, we overexpressed calumenin by genetic manipulation using an adenovirus gene transfer system in NRVCs to investigate the effects of overexpressed calumenin on ERS-mediated signaling cascades. As shown in Figs. 2 and Fig. S2, the overexpression of calumenin significantly downregulated ERS-mediated signaling proteins such as GRP78, p-PERK, and p-eIF2α, an observation which is similar to those reported in previous studies showing attenuation of ERS through various means [17–19,27]. In the UPR pathways, GRP78 dissociates from PERK, allowing its phosphorylation and activation. PERK is a serine/threonine kinase that catalyzes the phosphorylation of eIF2α to shut off mRNA

translation and reduce the protein load on the ER [6,7]. According to the results of the present study, overexpression of calumenin decreased the levels of p-PERK and p-eIF2α (Figs. 2 and Fig. S2), suggesting that UPR is diminished by calumenin overexpression, resulting in reduced amounts of unfolded proteins in the ER.

The increased expression of chaperones has been shown to protect cells against ERS-induced apoptosis [8,19,27]. Our results showed that the apoptosis markers CHOP and p-JNK were upregulated by TM or Tg induction, but their expression levels were decreased in Ad-calu–infected cardiomyocytes. Conversely, the level of anti-apoptotic marker Bcl-2 was reduced by TM or Tg induction, but it was increased in Ad-calu–infected cardiomyocytes. It has been reported that activation of the PERK-ATF4-CHOP branch of the UPR triggers pro-apoptotic signals [6]. Based on our Western blotting results that showed reduced levels of p-PERK, p-eIF2α and their downstream molecule CHOP caused by calumenin overexpression, calumenin acts as a putative regulator of PERK signaling and may therefore alleviate apoptosis. Moreover, the results of the TUNEL assay in this study revealed that calumenin overexpression reduced the number of apoptotic cells, suggesting a survival effect of calumenin against ERS-initiated apoptosis.

Most ER-residing chaperones are high-capacity and low-affinity Ca^{2+}-binding proteins with their functions regulated by the concentration of ER luminal Ca^{2+}. It has been shown that the activities of GRP78, calreticulin, and calnexin are decreased by low Ca^{2+} concentration in the ER lumen [28,29]. Indeed, ER Ca^{2+} concentration can regulate the interactions of chaperones with their substrate proteins or with other chaperones. For example, at low luminal Ca^{2+} concentrations, the interaction between calreticulin and PDI is promoted, so the activity of PDI is thereby reduced [30]. Since calumenin is a multiple EF-hand Ca^{2+}-binding protein in the lumen of ER/SR that is similar to other chaperones, the Ca^{2+}-dependent regulation of calumenin for ERS may be an interesting topic for future investigation.

In conclusion, our results showed that overexpression of calumenin significantly alleviated ERS and inhibited the ER-initiated apoptotic process, suggesting that calumenin may be an important molecular chaperone in NRVCs. Based on the observation that UPR signaling is associated with various other pathophysiological pathways, such as the pathways for cardiac hypertrophy and heart failure, calumenin may serve as a therapeutic target against various heart diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.087.

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