A novel endoplasmic reticulum stress-induced apoptosis model using tunicamycin in primary cultured neonatal rat cardiomyocytes

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Abstract. Endoplasmic reticulum (ER) stress is key in the development of cardiovascular diseases. However, there is a lack of a systemic ER stress-induced cardiomyocyte apoptosis model. In the present study, primary cultured neonatal rat cardiomyocytes were exposed to tunicamycin. Cell viability was determined by an MTT assay, and cell damage was detected by a lactose dehydrogenase assay. Flow cytometry was used and the activity of caspase-3 was analyzed in order to measure apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blotting were used to examine the expression of glucose-regulated protein 78-kDa (GRP78) and C/EBP homologous protein (CHOP). As a result, tunicamycin significantly increased cardiomyocyte injury, which occurred in a time- and concentration-dependent manner. In addition, tunicamycin treatment resulted in apoptosis of cardiomyocytes. Molecularly, tunicamycin (100 ng/ml) increased the levels of GRP78 and CHOP 6 h after administration. In addition, GRP78 and CHOP reached maximum mRNA and protein levels 24 h after administration. In conclusion, the results implicate that the tunicamycin-induced ER stress-induced apoptotic model was successfully constructed in cultured neonatal rat cardiomyocytes. A 100 ng/ml concentration of tunicamycin was selected, and MTT, LDH release and flow cytometry assay was at 72 h. In addition, GRP78 and GRP94 were detected 24 h following administration. The results of the present study indicate a novel experimental basis for the investigation of ERS-induced cardiac apoptosis.

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Introduction

The endoplasmic reticulum (ER) is one of the largest cellular organelles and has diverse functions, such as regulating the folding of membrane proteins and secretory proteins (1). Various stimuli, such as ischemia (2), hypoxia (3), heat shock, genetic mutation (4), oxidative stress (5) and elevated protein synthesis could result in ER dysfunction. Stresses that lead to the impairment of ER function are collectively known as ER stress (6,7). ER stress triggers an evolutionarily conserved response termed the unfolded protein response (UPR), an adaptive mechanism that initially promotes organelle recovery (8). In response to ER stress, there is significant upregulation of various ER resident chaperones, such as glucose-regulated protein 94-kDa (GRP94) and glucose-regulated protein 78-kDa (GRP78) that inhibit protein synthesis and activate protein degradation (9-10). When ER stress is excessive and/or prolonged however, apoptotic cell death is triggered by transcriptional induction of C/EBP homologous protein (CHOP) and/or by the activation of c-JUN NH2-terminal kinase (JNK), and/or caspase-12-dependent pathways (11).

Accumulating evidence demonstrates that ER stress-induced apoptosis is the key contributor to cell loss in the pathogenesis of a series of cardiovascular diseases, such as ischemia/reperfusion heart diseases (12,13), atherosclerosis (6,14,15), acute coronary syndrome (16), myocardial infarction (17,18) and heart failure (19,20). Tunicamycin acts as a highly specific ER stress inducer by inhibiting N-linked glycosylation of protein. Since there is a lack of a systemic ER stress-induced apoptotic model in cardiomyocytes, the present study constructed an endoplasmic reticulum stress-induced apoptotic model using tunicamycin in primary cultured rat neonatal cardiomyocytes. The optimal treatment time and

concentration of tunicamycin in cardiomyocytes was investigated. Cell viability was detected using an MTT assay and cell damage was observed using an LDH release assay. Apoptosis was measured using a flow cytometry assay and determining the activity of caspase-3. Finally, the expression of ER stress markers, including GRP78 and CHOP was induced by tunicamycin at different time points in cardiomyocytes.

Materials and methods

Reagents and antibodies. Tunicamycin, collagenase I, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH), dimethyl sulfoxide (DMSO), 5-bromo-2-deoxyuridine (BrdU) and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tunicamycin was dissolved in DMSO. Dulbecco's modified Eagle's medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Anti-GRP78 antibody was obtained from Bioworld (St. Louis Park, MN, USA). Anti-CHOP and anti-β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat anti-rabbit and goat anti-mouse antibodies conjugated to IRDyeTM^{800} (Rockland Inc., IL, USA) were detected using an Odyssey infrared imaging system (LI-COR Inc., Lincoln, NE, USA).

Culture of primary neonatal rat cardiomyocytes. Primary culture of neonatal rat cardiomyocytes was prepared with the use of 53 neonatal Sprague-Dawley rats from the Fourth Military Medical University (Xi'an, China), as described previously (21). In brief, neonatal rat ventricles were digested with collagenase I and cardiomyocytes were purified by 1 h incubation at 37˚C in a 5% CO_2 incubator. Cardiomyocytes were cultured in DMEM medium (containing 0.1 mmol/l BrdU) with 10% FBS. Cardiomyocytes amounted for 90-95% of total adherent cells and then were treated with tunicamycin at multiple concentrations (25, 50, 100, 200 and 500 ng/ml) and time-points (24 , 48 , 72 and 96 h).. All procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996), and approved by the Fourth Military Medical University Committee on Animal Care (Xi'an, China).

Cell viability determined by an MTT assay. Cell viability was assessed by an MTT assay as described previously (22). Briefly, cardiomyocytes were seeded in 96-well plates at a density of 5x10^4/well. After tunicamycin administration at the concentrations and durations, described above, MTT solution (10 μl, 5 mg/ml in PBS) was added to each well and incubated at 37˚C for 4 h. Then, the medium was replaced by 150 μl DMSO per well. The plate was gently shaken for 5 min to completely dissolve the precipitate. The absorbance was measured at 490 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). Cell viability was expressed as a percentage of the control.

LDH release assay. To determine cardiomyocyte injury, LDH release in the medium was detected as described previously (21). The LDH release level was expressed as the rate of LDH released in the medium to total cellular LDH.

Apoptosis analysis by flow cytometry assay. An Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Sigma-Aldrich) was used to detect apoptosis as described previously (23). Following treatment, cardiomyocytes were washed twice with cold PBS and resuspended in binding buffer. FITC-Annexin V and propidium iodide were added according to the manufacturer's instructions. The mixture was incubated for 10 min in the dark at room temperature and then cellular fluorescence was measured with a FACSscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Annexin V labeled with a fluorophore could identify cells in the early stage of apoptosis, and PI was responsible for staining cells in the medium at late stages of apoptosis. The apoptotic rate was calculated as the percentage of Annexin V-positive and PI-negative cells divided by the total number of cells in the gated region.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cardiomyocytes were exposed to tunicamycin at the corresponding concentrations and time-points, following which the cells were removed through scraping with a cell scratch and the addition of TRIzol to lyse the cells. cDNA synthesis was performed using a QuantiTect Reverse Transcription kit with 1 μg total RNA (Takara Bio, Inc., Shanghai, China). PCR was performed on a Bio-Rad system using Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA). cDNA was diluted at 1:5 for each reaction and all qPCR performed using SYBR Green. The reaction conditions were 10 min at 95°C, and then 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Rat β-actin was used to normalize sample amplification. The following primer sequences were used: GRP78, forward: 5'-CTACCGGGACGAGGTACTGG-3' and reverse 5'-GGAAAAGGCGGTGAGGACTT-3'; CHOP, forward: 5'-CGGAGTGTACCCAGCACCATCA-3' and reverse 5'-CCCTCTCCTTTGGTCTACCCTCA-3'; and β-actin. β-actin, forward 5'-AGAGGGAAATCGTGCGTGAC-3' and reverse 5'-TTCTCCAGGGAGGAAGAGGAT-3'.

Western blot analysis for GRP78 and CHOP. Cardiomyocytes were lysed using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) in combination with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Electrophoresis and immunoblotting were conducted as described previously (24). In brief, following treatment with tunicamycin, cardiomyocytes were washed three times with ice-cold PBS and isolated. Proteins were extracted from cardiomyocytes and protein concentrations were determined using a BCA protein assay kit. Protein samples were loaded on 12% SDS-PAGE gels (Beyotime Institute of Biotechnology), and transferred onto nitrocellulose membranes (Pierce Biotechnology, Inc., Rockford, IL, USA). Following blocking with 5% non-fat milk in TBS containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature, protein bands were reacted with rabbit anti-rat GRP78 antibody (dilution, 1:1,000; cat. no. BS1154; Bioworld), rabbit anti-rat CHOP antibody (dilution, 1:500; cat. no. sc-575; Santa Cruz Biotechnology, Inc.) or mouse anti-rat β-actin

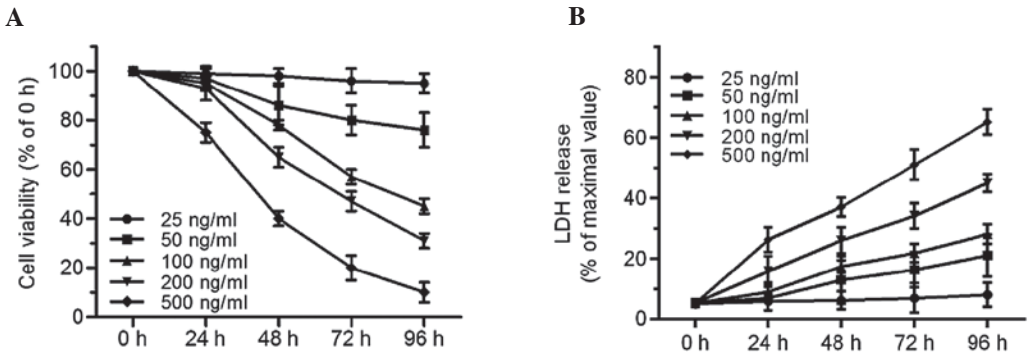

Figure 1. Tunicamycin induces cell injury in primary cultured neonatal rat cardiomyocytes. Cardiomyocytes were treated with tunicamycin (25, 50, 100, 200 and 500 ng/ml) for the indicated times (0, 24, 48, 72, 96 h). (A) Cell viability was measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and (B) cell injury was analyzed by the LDH release assay. For cell viability, the viable cell number was expressed as a percentage of the 0 h group. For the LDH release assay, all values were compared with the 0 h group. n=5. LDH, lactose dehydrogenase.

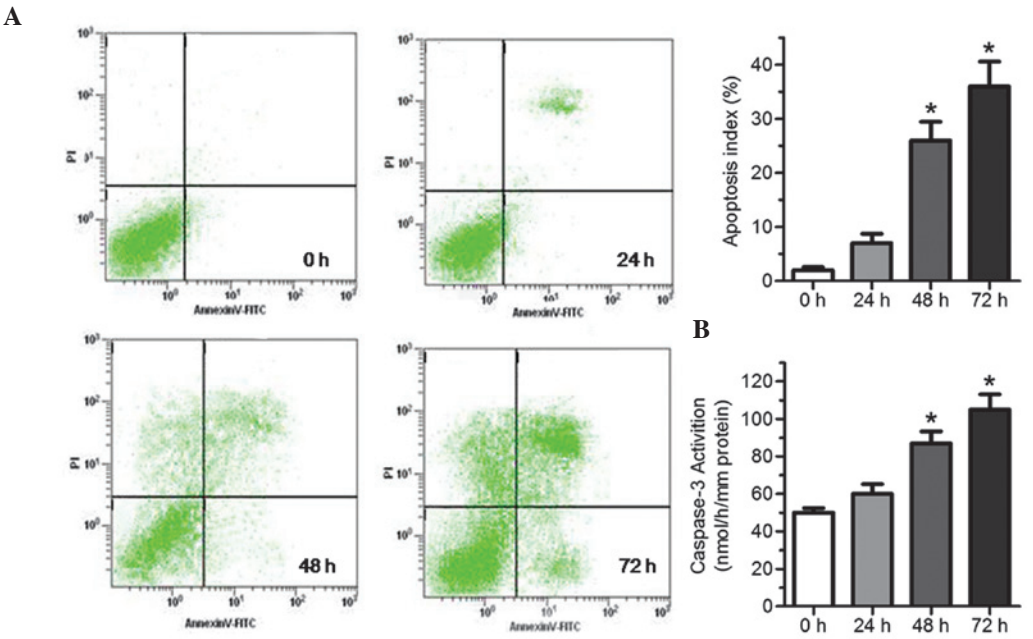

Figure 2. Tunicamycin-induced cell injury manifested as apparent apoptosis in primary cultured neonatal rat cardiomyocytes. Cardiomyocytes were treated with 100 ng/ml tunicamycin for the indicated times (0, 24, 48, 72 h). (A) Cardiomyocyte apoptosis was detected by flow cytometry. Quantitative analysis of apoptotic-positive cardiomyocytes. (B) The activity of caspase-3 in cardiomyocytes treated 100 ng/ml tunicamycin for the indicated times (0, 24, 48, 72 h). *P<0.05 vs. 0 h, n=5.

antibody (dilution, 1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc) in TBS overnight at 4˚C. Following washing three times with TBST, the membranes were hybridized with goat anti-rabbit or goat anti-mouse DyLIGHT^{800} antibodies for 1 h at room temperature. Following three washes with TBST, protein bands were visualized on infrared image system (Odyssey; LI-COR Biosciences, Inc., Lincoln, NE, USA). For the densitometry analysis, optical density was measured on the inverted digital images using Image J 1.46 software.

Statistical analysis. All experiments were performed at least five times. Data are expressed as the mean ± standard error of the mean. The results were compared by one-way analysis of variance followed by Bonferroni's test. P<0.05 was considered to indicate a statistically significant difference.

Results

ER stress inducer tunicamycin results in cardiomyocyte injury. An MTT assay and LDH release assay were used to evaluate cell injury in tunicamycin-treated cardiomyocytes. Tunicamycin, a pharmacological agent inhibiting N-linked protein glycosylation, could be used to experimentally induce ER stress and subsequent cell death. Concentrations of 25-500 ng/ml tunicamycin were selected. Compared with the control group, 25 ng/ml tunicamycin for 24-96 h had no effect on cell injury. However, cell viability decreased, and LDH release increased after 48-96 h exposure to tunicamycin at concentrations of 50, 100, 200 and 500 ng/ml (Fig. 1A and B). Treatment with 100 ng/ml tunicamycin for 72 h resulted in a decrease of cell viability to 57.4±3.2%. These results provide direct evidence

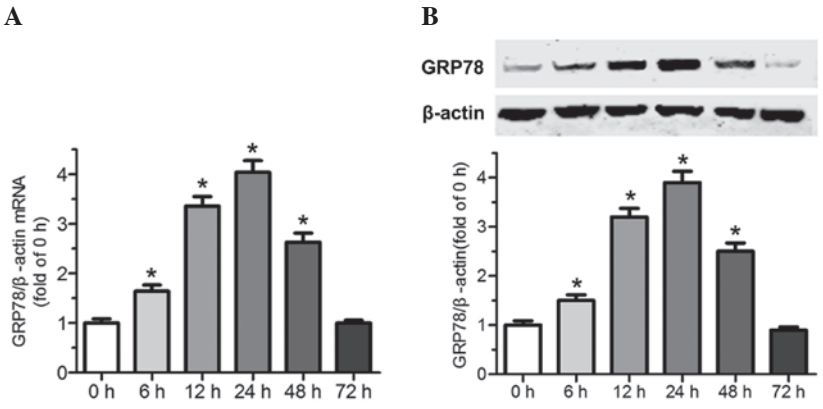

Figure 3. Tunicamycin induced upregulation of the ER chaperone GRP78 in cardiomyocytes. Cardiomyocytes were treated with 100 ng/ml tunicamycin for the indicated times (0, 6, 12, 24, 48, 72 h). (A) The expression of GRP78 mRNA levels was examined by RT-reverse trasncription-quantitative polymerase chain reaction. β-actin served as an internal control. (B) The expression of GRP78 at protein levels was examined by western blot analysis. β-actin served as a loading control. *P<0.05 vs. 0 h, n=5.

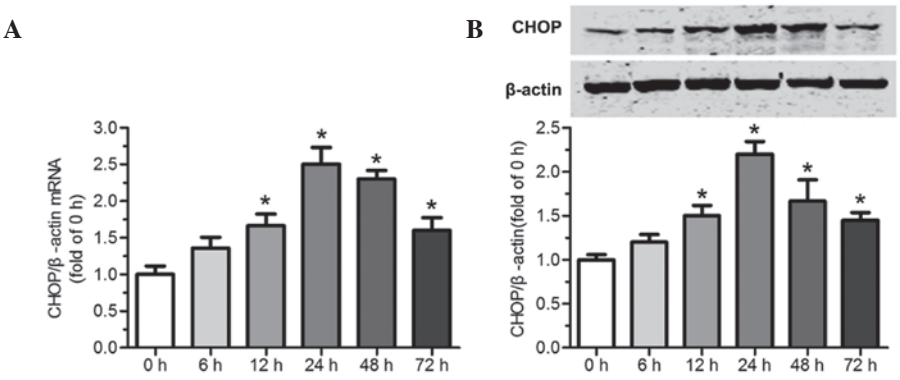

Figure 4. Tunicamycin induced expression of CHOP in cardiomyocytes. Cardiomyocytes were treated with 100 ng/ml tunicamycin for the indicated times (0, 6, 12, 24, 48, 72 h). (A) The expression of CHOP mRNA levels was examined by reverse transcription-quantitative polymerase chain reaction. β-actin served as an internal control. (B) The expression of CHOP at the protein level was examined by western blot analysis. β-actin served as a loading control. *P<0.05 vs. 0 h, n=5.

that tunicamycin led to significant cell injury in cardiomyocytes in a time- and dose-dependent manner.

Tunicamycin-induced cardiomyocyte injury manifested as apparent apoptosis. To assess the feature of cell injury induced by tunicamycin, a flow cytometry assay and analysis of caspase-3 activity were used to detect apoptosis. Compared with the control group, treatment with 100 ng/ml tunicamycin for 24 h did not induce marked apoptosis, as observed from flow cytometry assays (Fig. 2A) and activity of caspase-3 (Fig. 2B), whereas treatment with 100 ng/ml tunicamycin for 48-96 h significantly increased apoptosis (Fig. 2A), and activity of caspase-3 (Fig. 2B), particularly for 72 h.

Tunicamycin induces upregulation of ER chaperone GRP78 in cardiomyocytes. To confirm that tunicamycin induces ER stress, endoplasmic reticulum chaperone GRP78 was assessed by RT-qPCR and western blot analysis. As shown in Fig. 3A and B, there was a relatively low level of GRP78 in normal cardiomyocytes. However, treatment with tunicamycin (100 ng/ml) upregulated GRP78 at the mRNA and protein levels. The mRNA and protein levels of GRP78 began to increase following 6 h exposure to tunicamycin, it was then markedly upregulated and reached the maximum at 24 h. Subsequently, its expression significantly declined and returned to the basal level at 72 h. These results indicate that exposure of cardiomyocytes to tunicamycin induces upregulation of the ER resident molecular chaperone GRP78 at the mRNA and protein levels, and induces ER stress.

Tunicamycin induces CHOP expression in cardiomyocytes. To elucidate that tunicamycin induces the expression of CHOP in cardiomyocytes, CHOP levels were assessed by RT-qPCR and western blot analysis. As shown in Fig. 4A and B, there was relatively low expression of CHOP in normal cardiomyocytes. However, treatment with tunicamycin (100 ng/ml) increased CHOP at the mRNA and protein levels. CHOP levels began to increase at 6 h and reached a peak 24 h after exposure to 100 ng/ml tunicamycin. Subsequently, its expression slowly declined. The results provide evidence that tunicamycin triggers induction of CHOP in primary cultured neonatal rat cardiomyocytes.

Discussion

In the present study, an endoplasmic reticulum stress-induced apoptotic model was established in primary cultured neonatal rat cardiomyocytes. Firstly, it was demonstrated that

tunicamycin resulted in cardiomyocyte injury in a time- and dose-dependent manner. Secondly, apoptosis was the predominant mode of cell death of cardiomyocytes induced by tunicamycin. Thirdly, tunicamycin upregulated a number of ER stress markers, such as the survival/rescue protein GRP78 and the apoptotic molecule CHOP.

Accumulating evidence demonstrates that ER stress-induced apoptosis is the key contributor to cell loss in the pathogenesis of a series of cardiovascular diseases (8,25,26). However, the detailed mechanism of ER stress in cardiovascular diseases remains unclear. Tunicamycin is a specific inhibitor of N-linked glycosylation of protein, which is observed only in the endoplasmic reticulum, showing that tunicamycin is a highly specific ER stress inducer. Therefore, the present study aimed to establish an ER stress-induced apoptotic model using tunicamycin in primary cultured neonatal rat cardiomyocytes. Although tunicamycin has been used to induce ER stress in other cell types, cells are often regulated in a type- and stimulus-specific manner (27,28). Therefore, in order to screen the optimal treatment time and concentration of ER stress-induced cell injury by tunicamycin in cardiomyocytes, cell viability was assessed by an MTT assay, and cardiomyocyte injury was detected using an LDH release assay.

However, the pattern of cardiomyocyte injury-induced by tunicamycin remains unclear. Flow cytometry and analysis of the activity of caspase-3 were conducted to quantify the number of apoptotic cells. The present data revealed that tunicamycin induced cardiomycyte apoptosis. In addition, treatment of primary cultured neonatal rat cardiomyocytes with 100 ng/ml tunicamycin for 72 h led to the maximum rate of apoptosis.

Next, the effect of 100 ng/ml tunicamycin on ER stress-related molecules in cardiomyocytes was investigated. RT-qPCR and western blot analysis revealed that tunicamycin increased GRP78 and CHOP levels, and induced ER stress in primary cultured neonatal rat cardiomyocytes, which is consistent with the results of a previous study (29). GRP78 survival/rescue protein of cardiomyocytes treated with 100 ng/ml tunicamycin was rapidly upregulated, peaked at 24 h, and then rapidly declined to near preinduction level at the mRNA and protein levels. In addition, the level of apoptotic ER stress-related molecule CHOP was low at the early phase, peaked 24 h following treatment with 100 ng/ml tunicamycin and then decreased slowly. These data indicated that GRP78 was rapidly upregulated in the early stages of tunicamycin-induced ER stress in cardiomyocytes, whereas CHOP gradually increased in the first 24 h. The levels of GRP78 then rapidly decreased, whereas CHOP was slowly downregulated by persistent tunicamycin-induced ER stress in cardiomyocytes. However, how ER stress integrates its cytoprotective and proapoptotic outputs to select between life or death cell fates remains unknown.

In conclusion, the present study successfully constructed an ERS-induced cardiomyocyte apoptotic model with tunicamycin, and screened the optimal concentration and processing time for detecting cell viability, apoptotic index and ERS associated proteins GRP78 and CHOP. The results of the present study provide important experimental data for preclinical and clinical investigations of ERS-associated cardiovascular diseases.

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