Original Research

Endoplasmic reticulum stress and C/EBP homologous protein-induced Bax translocation are involved in angiotensin II-induced apoptosis in cultured neonatal rat cardiomyocytes

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Abstract

The aim of this study was to identify the roles and potential mechanisms of endoplasmic reticulum stress (ER stress), proapoptotic transcription factor C/EBP homologous protein (CHOP) and Bax in angiotensin II (Ang II)-induced cardiomyocyte apoptosis. Cultured neonatal rat cardiomyocytes were incubated with Ang II or antisense CHOP oligonucleotide which was used to inhibit CHOP expression. Expressions of ER chaperone immunoglobulin heavy chain-binding protein (BiP), CHOP and cytochrome *c* were examined by Western blotting. Mitochondrial membrane potential (MMP) was detected by a spectrofluorimeter. Apoptosis was analyzed with flow cytometry. Bax translocation was determined by double-labeling of immunofluorescence and Western blotting. Our results showed that Ang II-induced cardiomyocyte apoptosis was associated with the upregulations of BiP and CHOP, Bax translocation, MMP deplorization and cytochrome *c* release. These above effects were suppressed by antisense CHOP oligonucleotide. Furthermore, BiP and CHOP expressions, reactive oxygen species (ROS) production and cardiomyocyte apoptosis, which were upregulated by Ang II, were depressed by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor apocynin. From our results, ROS, ER stress and CHOP-mediated Bax translocation may be involved in Ang II-induced cardiomyocyte apoptosis.

Keywords: angiotensin II, cardiomyocyte apoptosis, endoplasmic reticulum stress, CHOP, bax

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Introduction

Angiotensin II (Ang II) is one of the most important factors contributing to cardiomyocyte apoptosis in myocardial infarction.[1–3] Moreover, cardiomyocyte apoptosis may have effects on cardiac contractility, conduction disturbances, cardiac remodeling and dysfunction.[4,5] Thus, identification of the underlying mechanisms of Ang II-induced cardiomyocyte apoptosis is important for clinical applications.

The endoplasmic reticulum (ER) is an organelle responsible for the synthesis, initial post-translational modification, and proper folding, sorting and export of secretory and membrane proteins. It is known that the ER is sensitive to homeostasis alterations induced by a variety of stimuli, such as inhibition of *N*-linked glycosylation,[6] perturbation of calcium homeostasis,[7] nutrient deprivation[8] and exposure to free radicals.[6] Under these conditions, ER functions are disturbed and ER stress is induced. To survive under ER stress, cells equip themselves with self-protective mechanisms (termed 'unfolded protein response') which can rapidly attenuate general protein synthesis, induce the expression of ER chaperone proteins, and enhance the degradation of malfolded proteins.[9] However, if these adaptive responses are not sufficient to relieve the ER stress, the cell dies through apoptosis.[10,11] One of the most important apoptotic pathways activated by ER stress involves the C/EBP homologous protein (CHOP), also called gadd 153 (growth arrest and DNA damage-inducible gene 153), a proapoptotic transcription factor expressed at very low levels under physiological conditions, but strongly up-regulated in response to ER stress.[12,13] ER is highly developed in cardiomyocytes. ER stress activation has been found in various cardiac diseases including myocardial ischemia, and CHOP has been reported to play a pivotal role in cardiomyocyte apoptosis.[14] Whether cardiomyocyte apoptosis induced by Ang II is associated with ER stress therefore has important clinical implications.

The mitochondrion is a critical site where various apoptotic signals converge. The Bcl-2 proto-oncogene family is critical for mitochondria-mediated apoptosis.[15,16] Bax is an important pro-apoptotic factor of this family.[3] In healthy cells, Bax is predominantly located in the cytoplasm. Upon apoptosis induction, Bax undergoes a conformational change and translocates from the cytosol to the mitochondria associated with its pro-apoptotic activity.[17,18] In previous studies, Bax has been shown to be upregulated in cardiac damage.[19] But, few studies have examined Bax subcellular localization during cardiomyocyte apoptosis. Although a previous study reported that CHOP expression results in downregulation of Bcl-2 expression,[20] the relationship of CHOP and Bax in cardiomyocytes stimulated by Ang II is unknown.

Therefore, the aims of this study were to investigate the role of ER stress in Ang II-induced cardiomyocyte apoptosis *in vitro* and to identify the potential mechanisms that may be responsible for the effect of ER stress. Here, we report that ER stress obviously contributed to cardiomyocyte apoptosis caused by Ang II. Furthermore, our results showed that CHOP induction was required for Bax translocation in response to Ang II treatment, and that reactive oxygen species (ROS) production mediated ER stress.

Materials and methods

Materials

This study complied with the Code of Ethics of the World Medical Association for the care and use of experimental animals, and was approved by the Animal Research Committee of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). One-day-old Wistar rats were obtained from the Experimental Animal Center of Tongji Medical College, certificate no 19–050. Ang II was obtained from Sigma-Aldrich (St Louis, MO, USA). Antibodies for BiP, CHOP, VDAC1, cytochrome *c*, Bax (Δ21), which reacts with any conformation of Bax in subcellular fractionation, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies for Bax (6A7) were purchased from Axxora Platform (San Diego, CA, USA). Antibodies for α-actin were purchased from Epitomics Inc. (Burlingame, CA, USA). All other chemicals used were of the highest grade available commercially.

Neonatal cardiomyocyte culture

Cardiomyocytes were obtained from one-day-old neonatal Wistar rats and cultured as previously described.[21] Minced ventricular myocardium was incubated into D-Hanks' salt solution, followed by digestion with 0.125% trypsin. After each of five successive eight-minute incubations, the dissociated cells were mixed with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and were centrifuged and pooled. The dissociated cells were enriched for cardiomyocytes by the technique of differential adhesion for 90 min and plated at a concentration of 10^6 cells/well. Cultures were incubated in a humidified 5% CO_2–95% O_2 atmosphere at 37°C. Bromodeoxyuridine (0.1 mmol/L) was added into the medium to inhibit proliferation of non-myocytes. This procedure yielded cultures with 90–95% myocytes, as assessed by microscopic observations of cellular contractions. After a overnight incubation in DMEM containing 10% FBS, the attached cells were rinsed and maintained in DMEM containing 0.1% FBS. All experiments were performed after 48 h of serum starvation.

Antisense treatment

An antisense CHOP oligonucleotide (CTGAGCCATAGAACTCTG) directed against the 50 coding region of the rat CHOP gene was synthesized (Sangon Co., Ltd, Shanghai, China). A missense oligonucleotide (ACTGCGCAATAAGGTCTC) served as the control, having an identical length, and containing the same nucleotide composition in a scrambled form. Both oligonucleotides contained phosphorothioate linkages to limit degradation. Transfection was performed one day before Ang II or Tg exposure using 5 μmol/L oligonucleotides and lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as indicated by the manufacturer. The efficiency of CHOP antisense to prevent CHOP expression was confirmed by reverse transcription-polymerase chain reaction at 18–48 h after transfection.

Preparation of cytosolic and membrane fractions

Following appropriate experimental treatment, cells were collected by centrifugation at $600 \times g$ for five minutes at 4°C, washed in ice-cold phosphate-buffered saline (PBS) and resuspended in the extraction buffer. Subcellular fractions were prepared according to the protocol of the Mitochondria/Cytosol Fractionation Kit (BioVision, Exton, PA, USA). The protein concentration of the subcellular extracts was determined by a Bradford-based assay, and the extracts were stored at −80°C until use.

Western blotting analysis

Myocytes were harvested and lysed in lysis buffer. Equal proteins were loaded in each lane, were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Membranes were blocked in 5% bovine serum albumin (BSA) and then incubated with primary antibodies for one hour. Thereafter, membranes were incubated with peroxidase-conjugated second antibody and then detected with enhanced chemiluminescence.

Immunofluorescence labeling

For double-labeling of Bax (6A7) and MitoTracker Red CMXRos, cells were cultured with 250 nmol/L of MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C prior to fixation in 4% paraformaldehyde and permeabilization with 0.25% Triton X-100. Cultures were then washed with PBS and incubated for one hour in 5% BSA, followed by three hours at room temperature in the presence of a mouse monoclonal

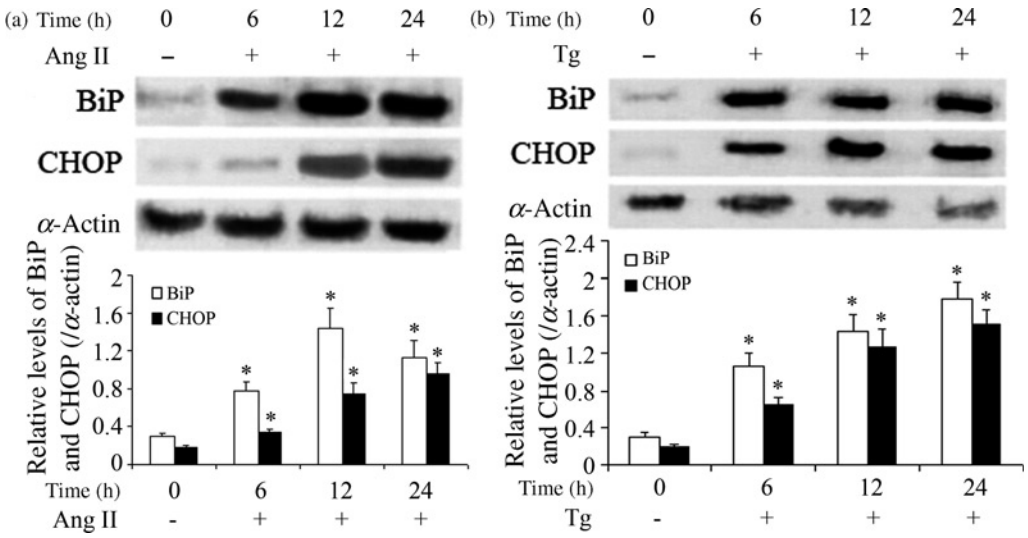

Figure 1 Induction of BiP and CHOP at protein levels. Cardiomyocytes were subjected to 100 nmol/L Ang II (a) or 2 μmol/L thapsigargin (Tg, b) for the indicated times. Expressions of BiP and CHOP in cardiomyocytes were detected by Western blotting. α-Actin served as a loading control. Relative expression levels of BiP and CHOP were normalized to α-actin. The experiments were repeated three times with reproducible results. Data represent means $\pm$ SD. $^{*}P < 0.05$ versus control. BiP, binding protein; CHOP, C/EBP homologous protein; Ang II, angiotensin II

anti-Bax (6A7) antibody diluted 1:500 in PBS. Fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (Zhongshan Biotech., Beijing, China) was then added and further incubated for 45 min at 37°C. After the slides were covered with glycerol buffer, the cells were observed under fluorescence microscopy and photos were taken.

Annexin V/propidium iodide staining

Cardiomyocytes were harvested and resuspended in binding buffer (containing 10 mmol/L HEPES/NaOH pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) followed by incubation with FITC-annexin in darkness for 10 min. The cells were then washed again in PBS, centrifuged and resuspended in binding buffer (100 μL). Propidium iodide (PI) was added at a final concentration of 2 mg/L and incubated for five minutes before immediate analysis of the cells on the flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Apoptotic cells were positive for annexin V and negative for PI.

Determination of mitochondrial membrane potential

A standard protocol was used in accordance with the instructions of the mitochondrial membrane potential (MMP) detection kit (Beyotime, Shanghai, China). Cardiomyocytes were harvested and resuspended in binding buffer, each sample was treated with 1 mL JC-1 and placed in a 37°C incubator for 20 min. The samples were then centrifuged at 600 *g* at 4°C for five minutes, washed twice with PBS, and resuspended in 200 μL of PBS. MMP was subsequently quantified on a fluorospectrophotometer (FP6300; Jasco, Tokyo, Japan). Data were collected at 530 nm emission for the JC-1 monomer and at 590 nm for JC-1 aggregates. Results were presented in relative aggregate to monomer fluorescence intensity ratio.

Measurement of ROS production

The determination of intracellular ROS production was based on the oxidation of 29,79-dichlorodihydrofluorescein (DCFH) to a fluorescent 29,79-dichlorofluorescein (DCF). Briefly, cardiomyocytes were loaded with 20 μmol/L DCFH (Molecular Probes) for 30 mins at 37°C in the dark. Cells loaded with DCFH were treated with 0.1 μmol/L Ang II in the presence or absence of propofol for 30 mins. Subsequently, the cells were washed twice with PBS, were detached by trypsin, and then were measured for DCF fluorescence intensity by fluorospectrophotometer analysis at an excitation wavelength of 488 nm and at an emission wavelength of 535 nm. The cell number in each sample was counted and was utilized to normalize the fluorescence intensity of DCF.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way analysis of variance. The differences were considered statistically significant at a value of $P < 0.05$.

Results

Ang II induces the ER stress involved in proapoptotic factor CHOP

To explore whether ER stress was linked to Ang II-induced cardiomyocyte apoptosis, we analyzed two known ER stress-specific proteins, namely, the ER chaperone BiP and the ER stress-associated proapoptotic factor CHOP,[22–24] using Western blotting (Figure 1a). The results of a time-course study indicated that the expression of BiP protein was at a low level before Ang II treatment. It increased at six hours after the treatment, and reached a maximum at 12 h. The expression of CHOP protein was examined as

well, and it was dramatically induced in a time-dependent manner by Ang II treatment. The level of CHOP protein in cardiomyocytes was very low under normal conditions, and began to elevate at six hours, lasting for up to 24 h after Ang II treatment, which is compatible with previous results.[2,3] If the regulation of these proteins by Ang II is linked to the ER stress response, a specific ER stressor, thapsigargin, should alter gene expression following a pattern similar to the one induced by Ang II. To test this hypothesis, cardiomyocytes were challenged with thapsigargin. It was found that both BiP and CHOP were up-regulated after six hours of thapsigargin treatment and the up-regulation lasted for up to 24 h (Figure 1b).

Disruption of the CHOP gene inhibits Ang II-induced cardiomyocyte apoptosis

To determine the role of CHOP in the cardiomyocyte apoptosis induced by Ang II, an antisense oligonucleotide against CHOP was used. In preliminary studies, we performed concentration–effect and time course analyses to establish conditions under which levels of CHOP proteins were maximally reduced. The results showed that a 24-h treatment period with 5 μmol/L antisense oligonucleotides resulted in a decrease in the level of the corresponding CHOP mRNA by about 55%. Oligonucleotide treatment and culture conditions were not cytotoxic to the cells, as assessed by MTT assay (data not shown). We therefore chose the antisense treatment conditions for all subsequent experiments. As shown in Figure 2a, the Western blotting showed that the levels of CHOP protein were markedly up-regulated in Ang II-treated cells compared with the control group, and CHOP antisense oligonucleotides suppressed the Ang II-induced CHOP overexpression. These results suggested that antisense oligonucleotide was an efficient blocker of CHOP expression. Furthermore, Ang II-induced apoptosis was also clearly suppressed after being treated with antisense oligonucleotide (Figure 2b). Moreover, the effects of CHOP antisense oligonucleotides on induction of apoptosis and the expression of CHOP by thapsigargin were also examined. As shown in Figures 2c and d, CHOP antisense oligonucleotides suppressed the thapsigargin-induced apoptosis and CHOP overexpression. Taken together, these observations support the notion that Ang II-induced apoptosis of cardiomyocytes involved the induction of CHOP.

Effects of CHOP on Bax translocation

Bax is a crucial player in cardiomyocyte apoptosis. Therefore, we assessed whether Ang II induced the activation and translocation of Bax and whether CHOP had any impact on that. We first analyzed activity-related conformational changes of Bax with the Bax(6A7) antibody recognizing N-terminal epitopes of Bax[25] and its mitochondrial localization with MitoTracker Red CMXRos by double-label immunofluorescence assay (Figure 3). In control cells, Bax-6A7 staining was undetectable. In Ang II-stimulated cells, the pattern of Bax-6A7 staining was markedly different from that in control cells. Bax-6A7 was co-localized with mitochondria marker, MitoTracker, as a punctuated staining pattern. This Bax staining pattern showed that Bax was activated and translocated to mitochondria under the Ang II stimulation. Cells transfected with the CHOP antisense oligonucleotides were resistant to Ang II-induced Bax N-terminal exposure and mitochondrial localization.

To confirm the immunofluorescence results, we further investigated the expression of Bax with Bax antibody (Δ21) by Western blotting. Bax was found predominantly in the cytosolic fraction of control cells (Figure 4), and it was nearly undetectable in the mitochondria-enriched heavy membrane fraction. In cells exposed to Ang II, the expression of Bax in the cytosolic fraction was depressed obviously, and that in the mitochondrial fraction was upregulated (Figure 4). This phenomenon was consistent with the immunofluorescence results, which indicated Bax translocation in neonatal cardiomyoctyes after Ang II stimulation. In contrast, Ang II-induced Bax translocation was restrained obviously in cells transfected with CHOP antisense oligonucleotides (Figure 4). Therefore, CHOP appeared to mediate Ang II-induced apoptosis by triggering the movement of Bax from the cytosol to the mitochondrial membrane.

Effects of CHOP on MMP and cytochrome *c* release

Bax localization to mitochondria is associated with loss of MMP and release of cytochrome *c*.[17,26] Next, we investigated the disruption of MMP. As shown in Figure 5, treatment of cardiomyocytes with Ang II caused a marked depolarization of MMP compared with the control. The CHOP antisense oligonucleotide significantly alleviated the depolarization of MMP caused by Ang II.

Bax interacts with components of the mitochondria permeability transition pore to promote opening of the pore, which ultimately causes cytochrome *c* release into the cytosol. Therefore, we assessed the effects of Ang II on cytochrome *c* releasing from mitochondria using Western blotting. Compared with control cells, in cells exposed to Ang II, the cytochrome *c* was elevated in the cytosolic fraction, and it was lessened in the mitochondrial fraction. Compared with cells treated with Ang II, the cytochrome *c* of cytosolic fraction was decreased obviously in cells transfected with CHOP antisense oligonucleotides, and that of mitochondrial fraction was increased markedly (Figure 6).

Apocynin abolishes ER stress and attenuates Ang II-induced cardiomyocyte apoptosis

To further understand the mechanisms by which Ang II induces ER stress in cardiomyocytes, we explored the role that ROS could play in this process. It is known that generation of ROS is regulated by various cytokines and growth factors, including Ang II, which causes $\cdot O_2^-$ and H_2O_2 production through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation in cardiomyocytes.[27–29] ROS generated from NADPH oxidase is a key step in Ang II-induced DNA damage and apoptosis.[27] To explore whether Ang II-induced ER stress depends on an increase

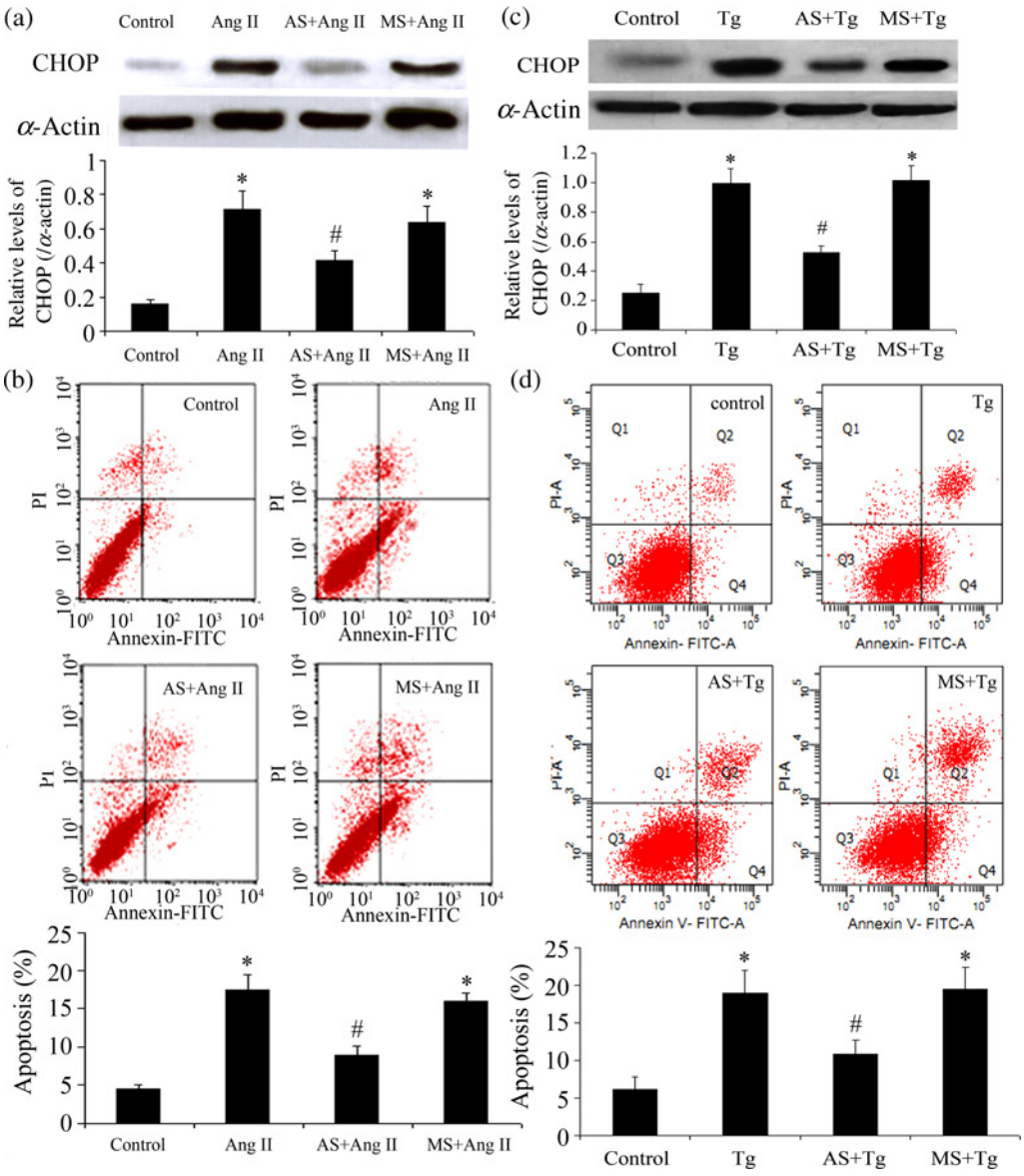

Figure 2 Effects of CHOP antisense phosphorothioate oligonucleotides on CHOP protein levels and apoptosis in Ang II or thapsigargin-stimulated cardiomyocytes. (a, c) Representative Western blotting results showed the protein expression levels of CHOP following indicated treatments. α-Actin served as a loading control. Relative expression levels of CHOP were normalized to α-actin. (b, c) Flow cytometer analysis of the cell apoptosis by FITC-conjugated annexin V and propidium iodide (PI) staining. Apoptotic cells were positive for annexin V and negative for PI. Control: cardiomyocytes were treated with culture medium only. Ang II: cardiomyocytes were treated with 100 nmol/L Ang II for 24 h. AS + Ang II: before 24 h Ang II (100 nmol/L) stimulation, cardiomyocytes were transfected with 5 μmol/L CHOP antisense phosphorothioate oligonucleotides (AS) for 24 h. MS + Ang II: before 24 h Ang II (100 nmol/L) stimulation, cardiomyocytes were transfected with 5 μmol/L CHOP missense phosphorothioate oligonucleotides (MS) for 24 h. Tg: cardiomyocytes were treated with 2 μmol/L thapsigargin (Tg) for 24 h. AS + Tg: before 24 h Tg (2 μmol/L) stimulation, cardiomyocytes were transfected with 5 μmol/L CHOP antisense phosphorothioate oligonucleotides (AS) for 24 h. MS + Tg: before 24 h Tg (2 μmol/L) stimulation, cardiomyocytes were transfected with 5 μmol/L CHOP missense phosphorothioate oligonucleotides (MS) for 24 h. The experiments were repeated three times with reproducible results. Data represent means ± SD. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus Ang II. FITC, fluorescein isothiocyanate; CHOP, C/EBP homologous protein; Ang II, angiotensin II. (A color version of this figure is available in the online journal)

of ROS, cardiomyocytes were pretreated with the NADPH oxidase inhibitor apocynin for 30 min and incubated with Ang II for indicated time. Subsequently, ROS production, BiP and CHOP expressions were determined. ROS formation was stimulated in cardiomyocytes after Ang II treatment for 30 min, and apocynin suppressed Ang II-induced ROS production (Figure 7a). In addition, Western blotting analysis revealed that pretreatment with 100 μmol/L apocynin for 30 min abrogated the Ang II-caused ER chaperon BiP and CHOP induction at 24 h (Figures 7b and c). To further confirm the role of ROS, we examined the effect of H_2O_2 on expressions of BiP and CHOP. Cardiomyocytes were treated with H_2O_2 (0.5 μmol/L) for 24 h. The results showed that H_2O_2 significantly increased expressions of BiP and CHOP in cardiomyocytes (Figures 7d and e), and antioxidant N-acetylcysteine (10 mmol/L) inhibited these effects of H_2O_2. In addition, annexin V/PI staining showed that apoptosis induced by Ang II was also blocked when cardiomyocytes were pretreated with apocynin (Figures 7f and g). Taken together, these data clearly demonstrated that intracellular ROS generation, ER stress and apoptosis in cardiomyocyte exposure to Ang II were closely linked.

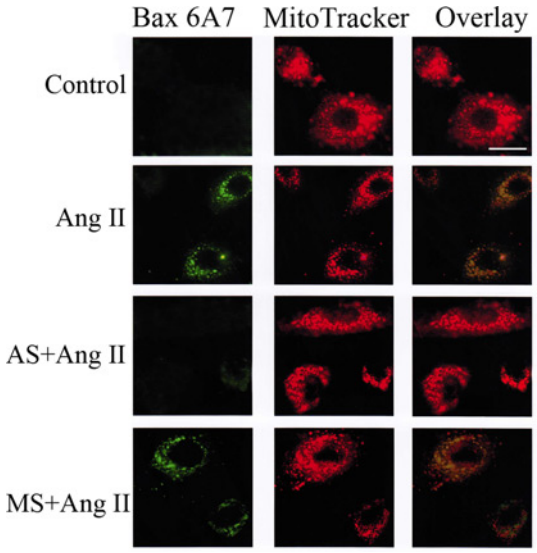

Figure 3 Effects of CHOP antisense phosphorothioate oligonucleotides on the Bax translocation in Ang II-treated cardiomyocytes. Before Ang II (100 nmol/L) stimulation, cardiomyocytes were transfected with either 5 μmol/L CHOP antisense (AS) or missense (MS) phosphorothioate oligonucleotides for 24 h. The treatments were the same as those in Figure 2. Cardiomyocytes were immunostained for Bax (green) and double-stained with mitotracker red for mitochondria (red). Bax and mitochondria localization were imaged by confocal microscopy. Co-localization of Bax in mitochondria was shown as yellow coloration in merged images. Scale bar, 10 μm. CHOP, C/EBP homologous protein; Ang II, angiotensin II. (A color version of this figure is available in the online journal)

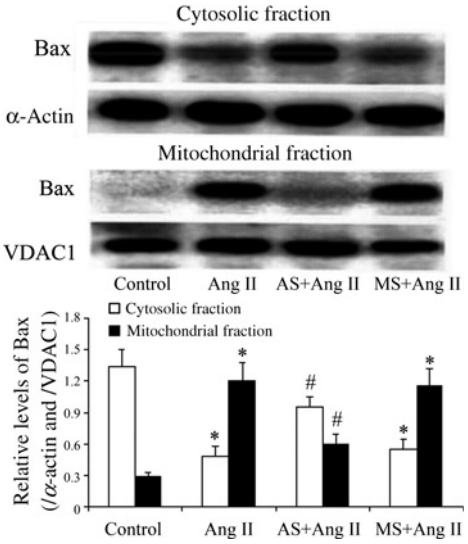

Figure 4 Effects of CHOP antisense phosphorothioate oligonucleotides on the expression of Bax in subcellular fraction. Before Ang II (100 nmol/L) stimulation, cardiomyocytes were transfected with either 5 μmol/L CHOP antisense (AS) or missense (MS) phosphorothioate oligonucleotides for 24 h. The treatments were the same as those in Figure 2. Bax expressions of cytosolic fraction and mitochondrial fraction were detected by Western blotting. α-Actin and VDAC1 were served as loading controls. Relative expression levels of Bax were normalized to α-actin and VDAC1. The experiments were repeated three times with reproducible results. Data represent means $\pm$ SD. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Ang II group. CHOP, C/EBP homologous protein; Ang II, angiotensin II

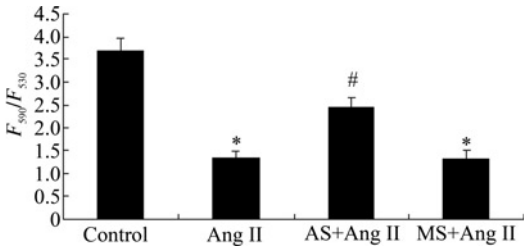

Figure 5 Effects of CHOP antisense phosphorothioate oligonucleotides on the depolarization of MMP in Ang II-treated cardiomyocytes. Before Ang II (100 nmol/L) stimulation, cardiomyocytes were transfected with either 5 μmol/L CHOP antisense (AS) or missense (MS) phosphorothioate oligonucleotides for 24 h. The results showed that CHOP antisense phosphorothioate oligonucleotides alleviated the dissipation of MMP induced by Ang II as studied spectrofluorometrically. The treatments were the same as those in Figure 2. The experiments were repeated three times with reproducible results. Data represent means $\pm$ SD. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus Ang II. CHOP, C/EBP homologous protein; Ang II, angiotensin II; MMS, mitochondrial membrane potential

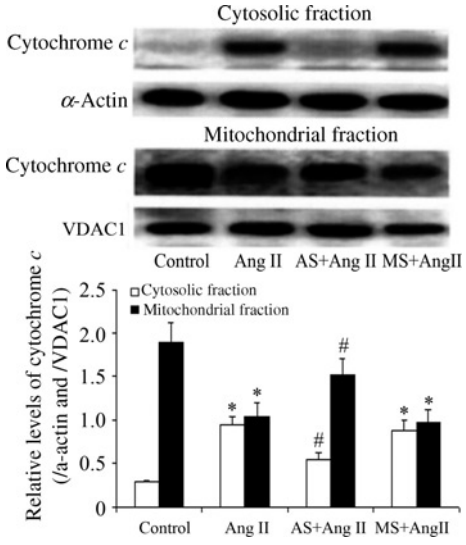

Figure 6 Effects of CHOP antisense phosphorothioate oligonucleotides on the releasing of cytochrome *c* in Ang II-treated cardiomyocytes. Before Ang II (100 nmol/L) stimulation, cardiomyocytes were transfected with either 5 μmol/L CHOP antisense (AS) or missense (MS) phosphorothioate oligonucleotides for 24 h. Cytochrome *c* expressions of cytosolic fraction and mitochondrial fraction were detected by Western blotting. α-Actin and VDAC1 served as loading controls. Relative expression levels of cytochrome *c* were normalized to α-actin and VDAC1. The treatments were the same as those in Figure 2. The experiments were repeated three times with reproducible results. Data represent means $\pm$ SD. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Ang II group. CHOP, C/EBP homologous protein; Ang II, angiotensin II

Discussion

ER stress and cell apoptosis have been studied extensively in various cell types. Recently, some studies have suggested that the participation of ER stress in apoptosis is associated with the development of heart diseases,[30] including ischemic heart diseases.[21] The ER chaperon BiP localizes in the ER, and its expression is up-regulated by environmental stressors in many types of cells. In the infarcted myocardium, the specific staining for BiP was robustly increased in tropomyosin-positive cells which may undergo

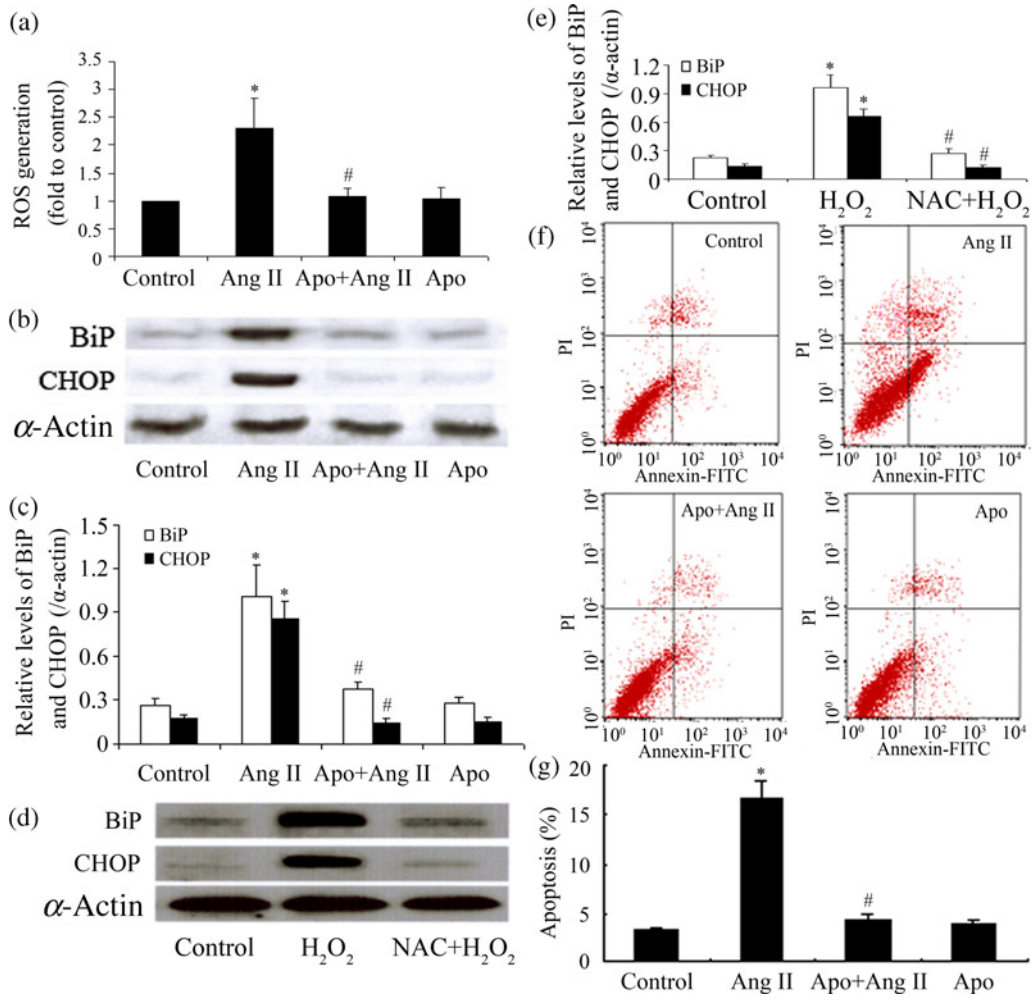

Figure 7 Effects of Apocynin on Ang II-induced ER stress and cardiomyocyte apoptosis. (a) Cardiomyocytes were pre-incubated with 100 μmol/L apocynin (Apo) for 30 min and stimulated with Ang II (100 nmol/L) for 30 min. ROS formation was analysed by fluorospectrophotometer. Results are expressed as the fold to control. (b, c) Cardiomyocytes were pre-incubated with 100 μmol/L apocynin (Apo) for 30 min and stimulated with Ang II (100 nmol/L) for 24 h. The expression of BiP and CHOP were detected by Western blotting. α-Actin served as a loading control. Relative expression levels of BiP and CHOP were normalized to α-actin. (d, e) Cardiomyocytes were treated with H_2O_2 (0.5 μmol/L) for 24 h with or without pretreatment of NAC (10 mmol/L) for 30 min. The expression of BiP and CHOP were detected by Western blotting. α-Actin served as a loading control. Relative expression levels of BiP and CHOP were normalized to α-actin. (f, g) Cardiomyocytes were pre-incubated with 100 μmol/L apocynin (Apo) for 30 min and stimulated with Ang II (100 nmol/L) for 24 h. Flow cytometer analysis of the cells apoptosis by FITC-conjugated annexin V and propidium iodide (PI) staining. Apoptosis cells were positive for annexin V and negative for PI. The experiments were repeated three times with reproducible results. Data represent means $\pm$ SD. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Ang II group. CHOP, C/EBP homologous protein; Ang II, angiotensin II; BiP, binding protein; FITC, fluorescein isothiocyanate; NAC, *N*-acetylcysteine. (A color version of this figure is available in the online journal)

apoptosis, and cardiomyocytes became resistant to lethal ischemic injury by pre-induction of BiP expression.[31] The inducer and signaling pathways of ER stress in ischemic cardiomyocyte apoptosis are still unclear. Ang II contributes to the development of apoptosis under the context of myocardial infarction. However, the underlying mechanisms are poorly defined. We found that BiP was strongly provoked by Ang II challenge. One potential mechanism for ER stress-induced apoptosis involves the participation of CHOP. Cells lacking CHOP are significantly protected from the lethal consequences of ER stress.[13,32] In our study, Ang II increased the expression of CHOP in a time-dependent manner. Moreover, abrogating the CHOP gene with CHOP antisense oligonucleotide, the Ang II-induced cardiomyocyte apoptosis was partially avoided, which indicated that CHOP played an important role in cardiomyocyte apoptosis, and that CHOP-independent and/or ER stress-independent apoptotic pathways could not be excluded.

Mitochondria and ER form endomembrane networks, and interact both physically and functionally.[33] To assess the role of the mitochondrial pathway in ER stress-mediated apoptosis caused by Ang II, we detected the translocation of pro-apoptotic molecule Bax, the change of mitochondrial membrane potential and release of cytochrome *c*, all of which were tightly related to the mitochondrial apoptotic cascade. In various cellular systems, Bax translocation to the mitochondria exerts a vital step in apoptosis. In normal conditions, Bax resides in the cytosol as a monomeric soluble protein; however, after apoptotic stress, it undergoes a conformational change exposing an N-terminal epitope, migrates towards mitochondria and

permeabilizes the mitochondrial outer membrane causing leakage of cytochrome *c*. Here, we also found that Ang II induced the activation and translocation of Bax associated with loss of mitochondrial membrane potential and cytochrome *c* release, by using anti-Bax 6A7 antibody which only recognizes the conformationally changed Bax protein. In addition, mouse embryo fibroblasts from $Bax^{-/-}Bak^{-/-}$ mice are resistant to apoptosis induced by ER stress, suggesting the role of Bax as executioner in ER stress-mediated apoptosis.[34] Subsequently, we found CHOP antisense oligonucleotides inhibited Bax translocation and alleviated the loss of mitochondrial membrane potential and releasing of cytochrome *c*, which were induced by Ang II. However, how CHOP contributes to the Bax conformational change remains unclear.

These results supported that regulation of Bax conformation mediated the process of CHOP-implicated ER stress in Ang II-induced cardiomyocyte apoptosis. Moreover, CHOP antisense oligonucleotides could only partially suppress the translocation of Bax, which indicated that other moleculars may be involved in Ang II-induced cardiomyocyte apoptosis besides CHOP. However, the partial suppression effect of CHOP antisense on Bax tanslocation could be related with the incomplete blocking effect of antisense oligonucleotides on CHOP expression (Figure 2a).

ROS can be generated in the cells through multiple sources, such as the electron transport chain in mitochondria, ionizing radiations,[35] lipoxygenases[36] and NADPH oxidases.[37] Ang II has been shown to stimulate the activation and expression of membrane-bound NADPH oxidase in cardiomocytes, thereby generating ROS and triggering apoptosis.[38] In our results, apocynin abolished the effects of Ang II on cardiomyocytes, indicating that Ang II provoked ER stress through the prior generation of ROS. The finding is compatible with previous reports showing that the ER is sensitive to oxidative stress.[39,40] Currently, little is known about how ROS induces ER stress. There are several explanations: the first possibility is that ROS may cause depletion of calcium stores in the ER via inhibition of Ca^{2+}-ATPase;[41] the second is that ROS may damage ER function by inducing the dysfunction of ER foldases and chaperones;[42] and the most important is that both ER and mitochondria can produce ROS,[43] and these two subcellular organelles are in such close proximity that they can influence each other's function with diffusible molecules, for example, ROS.[44]

In summary, the present study delivers important insights to the mechanisms underlying Ang II-induced cardiomyocyte apoptosis. Functional abnormalities of the mitochondria or ER are believed to be involved in cardiomyocyte apoptosis. However, little is known about their cooperation in the development of apoptosis induced by Ang II. We propose here, for the first time, a possible molecular mechanism on how ER stress and Bax translocation mediate the cardiomyocyte apoptosis stimulated by Ang II. The fact that Bax translocation is downstream of ER stress and CHOP expression implies that ER stress could trigger the mitochondrial apoptotic pathway. Moreover, intracellular ROS production mediates Ang II-associated induction of ER stress and apoptosis.

Conclusion

Our results support the hypothesis that Ang-II induces apoptosis and ER stress in cultured neonatal rat cardiomyocytes. Bax translocation is involved in this condition and CHOP may play a vital role in this process.

Author contributions: All authors participated in the design and execution of experiments and were directly involved with interpretation of the studies, analysis of the data and review of the manuscript.

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