

Upregulation of GRP78 and caspase-12 in diastolic failing heart

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Background: The endoplasmic reticulum (ER) fulfills multiple cellular functions. Various stimuli can potentially cause ER stress (ERS). ERS is one of the intrinsic apoptosis pathways and apoptosis plays a critical role in hypertension. Glucose regulated protein 78 (GRP78) has been widely used as a marker for ERS and caspase-12 mediated apoptosis was a specific apoptotic pathway of ER. The expression of GRP78 and caspase-12 remains poorly understood in the diastolic heart failure resulting from hypertension. **Methods:** We used spontaneously hypertensive rats (SHRs) to establish a model of diastolic heart failure, and performed immunohistochemistry, Western blot, and real-time PCR to analyze GRP78 and caspase-12. **Results:** We found that GRP78 and caspase-12 had enhanced expression at protein and mRNA levels. **Conclusions:** These results suggest that GRP78 and caspase-12 were upregulated in cardiomyocytes and ERS can contribute to cardiac myocyte apoptosis in the diastolic heart failure resulting from hypertension.

Keywords: apoptosis, caspase-12, diastolic heart failure, GRP78; hypertension

INTRODUCTION

Apoptosis is the key contributor to cell loss during heart failure (Chandrasekhar *et al.*, 2003; Wencker *et al.*, 2003). Interestingly, endoplasmic reticulum (ER) is an organelle involved in the intrinsic pathway of apoptosis (Ferri & Kroemer, 2001). The ER is recognized as an organelle that participates in folding secretory and membrane proteins (Kaufman, 1999; Ron, 2002). Various conditions can disturb the functions of the ER and result in ER stress (ERS). In response to ERS, there is a marked upregulation of ER chaperones such as glucose regulated proteins 78 and 94 (GRP78, GRP94) and calreticulin (Kaufman, 1999; Ferri & Kroemer, 2001; Patterson & Cyr, 2002).

Excessive and prolonged ERS triggers cell suicide. Cell apoptosis can proceed through obvious routes including activation of caspase-12, a representative caspase implicated in the cell death-executing mechanisms connected with ERS (Nakagawa *et al.*, 2000).

Emerging data indicate that apoptosis occurs in the critical organ (heart, brain, or kidney) in hypertension. It is well known that heart failure is the most frequent cause of death in hypertensive patients. However, the mechanisms by which ERS leads to cell death remain poorly understood, particularly in diastolic heart failure resulting from hypertension. So we examined the expression of GRP78 and caspase-12 in the hearts of spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats.

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Abbreviations: ATF6, transcription factor 6; CHOP, C/EBP homologous protein; ERS, endoplasmic reticulum stress; GRP78, glucose regulated protein 78; GRP94, glucose regulated protein 94; IHC, immunohistochemistry; IRE1, inositol requiring 1; JNK, c-Jun-N-terminal kinase; PBS, phosphate-buffered saline; PERK, PKR-like ER kinase; PMSF, phenylmethylsulfonyl fluoride; SHRs, spontaneously hypertensive rats; SPF, specific pathogen-free; TAC, transverse aortic constriction; TDT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated DNA nick-end labeling; UPR, unfolded protein response; WKY rat, Wistar-Kyoto rat.

MATERIALS AND METHODS

Preparation of animal model. Twenty SHR (8 weeks old, SPF, male) and twenty WKY rats (8 weeks old, SPF, male) were taken from vital river laboratories (Beijing Weitonglihua laboratories, China). The rats were housed in cages, and had free access to standard rat diet and tap water of the center. They were maintained under conditions of standard lighting (alternating 12 h light/dark cycle), temperature ($22 \pm 0.5^\circ\text{C}$) and humidity ($60 \pm 10\%$) for at least 1 week before the experiments. Then, ten SHRs or ten WKY rats were randomly killed in each group when they were 8 weeks or 32 weeks old. All the rats drank freely and their blood pressure was measured every 2 weeks.

Echocardiographic evaluation. Before sacrifice, each rat was anaesthetized with ketamine · HCl (50 mg/kg) and xylazine (10 mg/kg), and placed in the left lateral decubitus position. The chest of the rat was shaved and a layer of acoustic coupling gel was applied to the thorax. Two-dimensional and M-mode echocardiography was performed using a commercially available 12-MHz linear-array transducer system and echocardiogram machine (Sonos 5500, HP, MA, USA). M-mode recordings were obtained of the left ventricle at the level of the mitral valve in the parasternal view using two-dimensional echocardiographic guidance in both the short and long axis views. Pulsed-wave Doppler was used to examine mitral diastolic inflow from the apical four-chamber view. For each measurement, data from three consecutive cardiac cycles were averaged. All measurements were made from digital images captured at the time of the study by use of inherent analysis software (Sonos 5500 software package).

Immunohistochemistry (IHC) staining and terminal deoxynucleotidyl transferase-mediated DNA nick-end labeling (TUNEL) staining. Hearts were transversally sliced and fixed in 4% neutral formalin for over 24 h, and then embedded in paraffin, cut into 5 μm thick sections for IHC staining. The methods of the staining involved standard techniques as described previously (Li *et al.*, 2008). The tissue expression of GRP78 was assessed immunohistochemically using goat polyclonal anti-GRP78 antibody (diluted 1:200; Santa Cruz Biotechnology, CA, USA) and the sections incubated with PBS, instead of the primary antiserum, served as negative controls. The sections were examined using light microscopy.

TUNEL staining using the TUNEL Apoptosis Detection Kit (KGA 703, KeyGen Biotechnology, China) was performed according to the manufacturer's instructions. Briefly, sections were deparaffinized, digested with proteinase K (20 $\mu\text{g}/\text{ml}$) at room temperature for 15 min, and then washed in PBS for

5 min. Each section was covered with a TDT enzyme solution containing 45 μl equilibration buffer, 1 μl biotin-11-dUTP, 4 μl TDT enzyme, and incubated for 1 h at 37°C in a humidified chamber. After that, sections were soaked in stop buffer to terminate the enzymatic reaction, and then gently rinsed with PBS. A portion of 50 μl streptavidin-horseradish peroxidase (HRP) solution containing 0.25 μl streptavidin-HRP and 49.75 μl PBS was then applied to each section, and then incubated at room temp. for 30 min in the darkness. Slides were washed in PBS and exposed to 3,3-diaminobenzidine (DAB) chromogen for 5 min. The slides were then rinsed in water and counterstained with hematoxylin. Then the sections were examined using light microscopy. Sections incubated with PBS, instead of TDT enzyme solution, served as negative controls. The number of TUNEL-positive cells was counted in five randomly selected fields of view under 400 \times magnification for each animal, ten animals per group.

Real-time PCR. Total RNA was isolated from frozen renal tissues using Trizol reagent (Invitrogen, USA). The concentration of total RNA was quantified by spectrophotometry and reverse-transcribed with the use of the M-MLV Reverse Transcriptase System (Fermentas, Germany) and oligo(dT). Real-time PCR was performed with the use of LightCycler (Roche Applied Science, Germany) following the manufacturer's instructions. SYBR Green I kit (TaKaRa Biotechnology, Japan) was used and all the primers are listed in Table 1. The specificity of products generated for each set of primers was examined for each fragment with the use of a melting curve and gel electrophoresis. The relative expression levels of each target gene were normalized to the mRNA of the internal standard gene glyceraldehyde phosphate dehydrogenase (GAPDH).

Western blotting. Tissue samples from the hearts were homogenized in a buffer containing 20 mM Tris/HCl, pH 6.8, 1 mM EDTA, 1% SDS, 1 mM PMSF, and 1 \times protease inhibitor cocktail (Roche, Germany). Equal amounts of protein from each sample were separated by 10% or 12.5% SDS/PAGE as described previously. The separated proteins were transferred onto nitrocellulose membranes, and the membranes were blocked for 2 h with 5% defatted milk, then incubated overnight at 4°C with one of the following antibodies: GRP78, diluted 1:500, caspase-12 (Santa Cruz Biotechnology, CA, USA) diluted 1:600, cleaved-caspase-3 (Santa Cruz Biotechnology, CA, USA) diluted 1:500. After being washed, the membrane was incubated with the corresponding secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized with the SuperSignal West Pico enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Band inten-

Table 1. Oligonucleotide primer sequences and amplification conditions

	Primer sequences (5'-3')	Annealing temperature (°C)	Length (bp)
GRP78	*F AACCCAGATGAGGCTGTAGCA R ACATCAAGCAGAACCAGGTCAC	57	91
Caspase-12	F CACTGCTGATACAGATGAGG R CCACTCTTGCCTACCTTCC	56	138
GAPDH	F GTCGGTGTCAACGGATTG R ACAAACATGGGGGCA TCAG	59	397

*F, forward, R, reverse.

sities were quantified using a densitometer analysis system Flurochem 9900–50 (Alpha Innotech, USA). Equal protein loading was confirmed by staining the gel with Coomassie Blue and probing with β -actin antibody (Sigma, USA).

Statistical analysis. The experimental data were analyzed by using SPSS 11.5 software. All the results are expressed as mean \pm SEM. Individual groups were tested for differences by using one-way ANOVA repeated measurements, followed by independent samples *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Establishment of the model

Blood pressure and left ventricular mass index (LVMI, LVMI=left ventricular mass (LVM, mg)/body mass (BM, g)) differed significantly in the SHR animals compared with the sex-matched control WKY rats (Fig. 1). Echocardiographic data are shown in Table 2. Left ventricular diastolic function variables expressed by the ratio of E-wave (early diastolic filling, early peak velocity) and A-wave (late atrial filling, atrial peak velocity) differed significantly between SHRs (32 weeks) and WKY rats (32 weeks). A significant increase in the E-wave velocity, significant decrease in the A-wave velocity, and remark-

able increase in the E/A ratio was found ($E/A \gg 2$). These data demonstrate that the SHRs had diastolic heart failure at 32 weeks of age.

Localization of apoptosis by TUNEL assay and IHC analysis of GRP78 distribution

To assess whether hypertension results in apoptotic cell death in the hypertensive heart, the tissue sections were labeled with an TUNEL assay (Fig. 2). Apoptosis was observed in both cardio-myocytes and the endothelium of the hypertensive heart. More apoptotic cardiocytes were found for SHRs (32 weeks) groups. Estimation of cardiac apoptosis revealed a nearly threefold increase in TUNEL-positive nuclei in hypertensive heart. As can be seen in Fig. 3, immunochemistry studies showed that GRP78 was abundantly expressed in the myocardium of SHRs. In contrast, the myocardium from WKY rats group exhibited modest or weak immunoreactivity for this molecule. We also found that in this animal model, the increased tendency of GRP78-positive cells paralleled the increased tendency of apoptotic cells.

Western blot and RT-PCR analysis of GRP78 and caspase-12

On Western blots, we detected GRP78 and cleaved-caspase-3 proteins in rat myocardium as single bands migrating at 78 kDa and 17 kDa respectively, and two bands between 30–50 kDa for

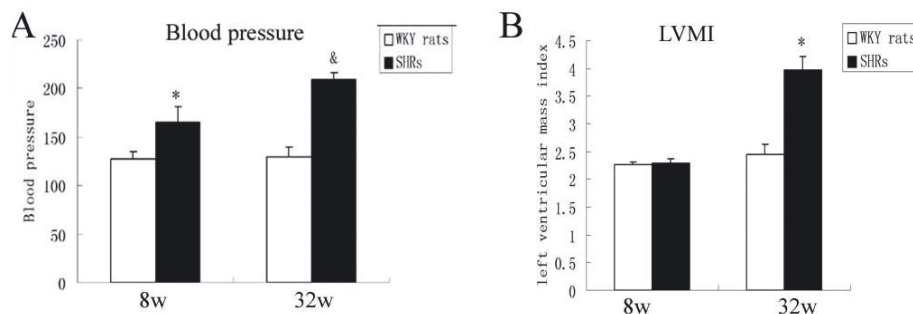


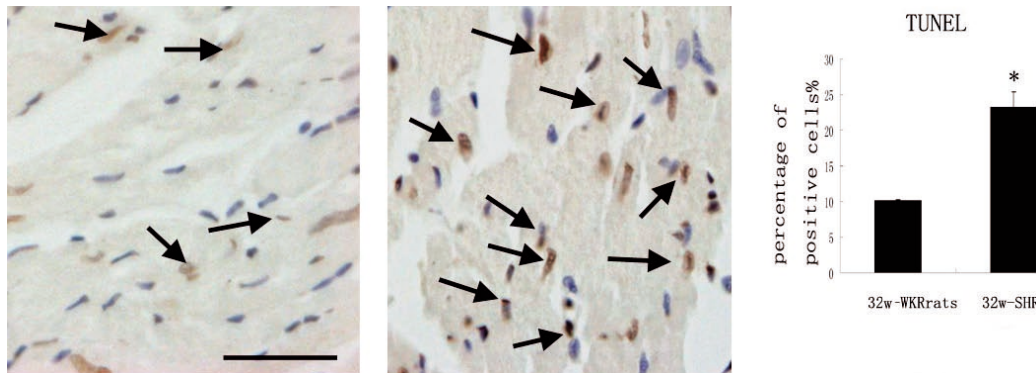
Figure 1. Blood pressure and LVMI in SHRs and WKY rats.

A. Blood pressure. B. LVMI. LVMI=left ventricular mass (LVM, mg)/body mass (BM, g). Ten animals were studied per group. * $P < 0.05$ vs WKY rats, & $P < 0.01$ vs WKY rats.

Table 2. Echocardiographic data

	8w-WKY rats	8w-SHRs	32w-WKY rats	32w-SHRs
Interventricular septum, mm	2.10±0.04	1.97±0.06	2.13±0.81	2.78±0.08*
Posterior wall, mm	1.7±0.07	2.4±0.16*	2.0±0.24	2.62±0.11*
E/A	1.57±0.11	1.49±0.06	1.38±0.04	2.46±0.20*
IRT	24.95±0.53	25.26±0.61	25.79±0.83	29.9±0.69*

Data are shown as mean±SEM. E: peak early transmitral filling velocity during early diastole; A: peak transmitral atrial filling velocity during late diastole; IRT: isovolumic relaxation time. EF: ejection fraction; FS: fractional shortening. Ten animals were studied per group. * $P<0.05$ vs WKY rats.

**Figure 2. TUNEL staining of apoptotic bodies (arrows).**

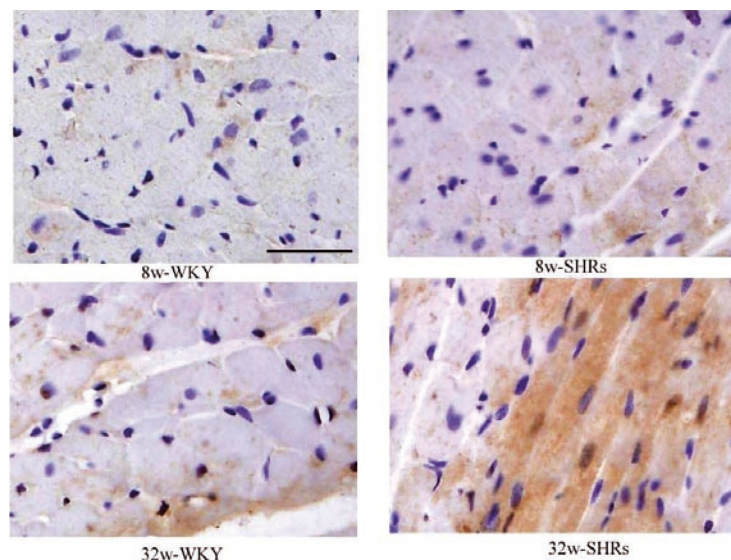
Compared with the normal heart, the hypertensive heart has notably higher expression. Quantitative analysis of TUNEL-positive cells as the percentage of total cell number revealed a nearly threefold increase in hypertensive heart (32 weeks). Ten animals were studied per group. * $P<0.05$ vs WKY rats. Bar=20 μ m (40 \times).

caspase-12. Coincident with the timing of the appearance of apoptosis (caspase-3 cleavage), the levels of ERS-associated proteins, including GRP78 and caspase-12, were markedly increased ($P<0.05$) in the hypertensive heart (Fig. 4).

The mRNA levels of the ERS-associated genes were measured by quantitative real-time PCR. mRNA levels of GRP78 and caspase-12 were found to be significantly ($P<0.05$) upregulated in the hypertensive heart, in parallel with their enhanced protein expression (Fig. 5).

DISCUSSION

The ER is a highly dynamic organelle that participates in the folding of secretory and membrane proteins. Several signaling pathways are initiated to cope with ERS, which are designated as the unfolded protein response (UPR) (Bernales *et al.*, 2006). One major pathway of UPR is to increase regulation of the expression of ER-localized molecular chaperons, such as GRP78, which can contribute to repairing unfolded proteins. Induction of GRP78 has

**Figure 3. Immunohistochemical staining of ER-stress associated protein GRP78.**

This protein locates in the cytoplasm of cardiocyte and it was abundantly expressed in the hearts of SHR (32 weeks) group. Bar=20 μ m (40 \times).

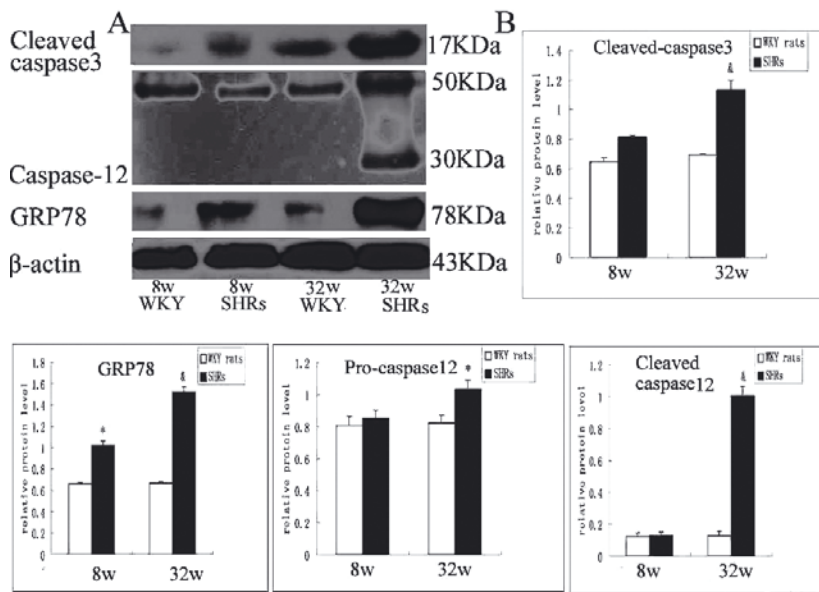


Figure 4. Upregulation of the ERS-associated proteins.

A. GRP78, caspase-12, and cleaved caspase-3 in SHRs and WKY rat hearts. B. Quantitative analysis of the ERS-associated proteins in rat hearts. Ten animals were studied per group. * $P < 0.05$ vs WKY rats, & $P < 0.01$ vs WKY rats. □ WKY rats, ■ SHR rats.

been widely used as a marker for ERS and the onset of UPR (Schröder & Kaufman, 2005). Furthermore, GRP78 serves as a master modulator for the UPR network by binding to the ERS sensors PKR-like ER kinase (PERK), inositol requiring 1 (IRE1), and transcription factor 6 (ATF6) and inhibiting their activation (Schröder & Kaufman, 2005). Increased GRP78 was reported in ERS-associated apoptosis of pancreatic β cells, renal proximal tubular cells (Ohse *et al.*, 2006), cardiocytes in the heart failure (Bhimji *et al.*, 1986), in diabetic cardiomyopathy (Li *et al.*, 2008), and in endothelial cells (Yoneda *et al.*, 2001).

In our present study, the mRNA and protein levels of GRP78 were markedly upregulated. This finding suggests that hypertension activates ERS in the rat heart. Emerging data has indicated that excessive and/or prolonged ERS leads to the initiation of the

apoptotic processes promoted by transcriptional induction of C/EBP homologous protein (CHOP) or by the activation of c-Jun-N-terminal kinase (JNK) and/or caspase-12-dependent pathway (Oyadomari *et al.*, 2002). Caspase-12 knockout mice exhibit resistance to ERS, suggesting that caspase-12 also plays a role in the process of cell death caused by ERS. Nakagawa and coworkers al demonstrated that caspase-12 mediated apoptosis was a specific apoptotic pathway of ER and m-calpain might be responsible for cleaving procaspase-12 (Nakagawa *et al.*, 2000; Rao *et al.*, 2002). Activated caspase-12 can trigger caspase-9, then procaspase-3 can be cleaved by activated caspase-9. Activated procaspase-3 would lead to the apoptosis of cells. Recent work has supported the claim that caspase-3 is an executioner of apoptosis induced by a variety of stimuli (Pytlowany *et al.*, 2008).

However, the mRNA and protein levels of caspase-12 were not upregulaed in the cardiomyocyte of SHRs (8 weeks), and this pathway was activated only in the SHRs (32 weeks) group. We conjecture that in the preliminary stage of hypertension, in order to block these stresses, the ER only switches on the UPR. The UPR will allow the cells to accommodate to the changed environment and reinstate the normal function of the ER. These adaptive mechanisms involve transcriptional programs that induce expression of genes that enhance the protein folding capacity of the ER, and promote ER-associated protein degradation to remove misfolded proteins (Xu *et al.*, 2005). Interaction between the chaperones and proteins ensures that only proteins that are properly assembled and folded leave the ER compartment and

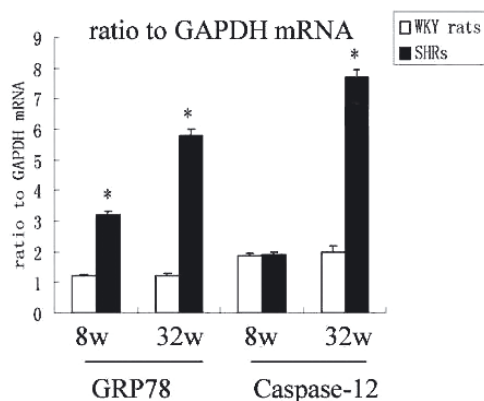


Figure 5. Upregulation of the ERS-associated genes.

Total RNA was extracted from myocardium and subjected to real-time PCR quantification as described under Materials and Methods. * $P < 0.05$ vs WKY rats, & $P < 0.01$ vs WKY rats.

thus attempt to alleviate the threat of cell death (Kaufman, 1999).

Recent studies have also demonstrated that the apoptosis of cardiocytes is connected with ERS. Studies in transverse aortic constriction (TAC) mice have demonstrated that pressure overload by TAC induces prolonged ERS, which contributes to cardiac myocyte apoptosis during the progression of cardiac hypertrophy to failure (Okada *et al.*, 2004). In the hearts of MCP mice (transgenic mice that express MCP-1 specifically in the heart) strongly suggest that activation of ERS response is involved in the development of ischemic heart disease (Azfer *et al.*, 2006). Studies in the mutant KDEL receptor transgenic mice found that the dilated cardiomyopathy is associated with ERS (Hamada *et al.*, 2004).

In conclusion, we can state that long-term hypertension has been shown to be an effective stimulus for cardiocyte apoptosis. If we could effectively prevent the cell death caused by ERS, we could reduce the damage to the critical organs resulting from hypertension. Further investigation of the intracellular signaling and the role of ERS induced apoptosis in hypertensive heart will be needed.

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