

## Partial Inactivation of Cardiac 14-3-3 Protein *in vivo* Elicits Endoplasmic Reticulum Stress (ERS) and Activates ERS-initiated Apoptosis in ERS-induced Mice

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### Key Words

14-3-3 • Endoplasmic Reticulum stress • Apoptosis  
• Pressure overload

### Abstract

**Background/Aims:** Excessive endoplasmic reticulum stress (ERS) triggers apoptosis in various conditions including diabetic cardiomyopathy and pressure overload-induced cardiac hypertrophy and heart failure. The primary function of 14-3-3 protein is to inhibit apoptosis, but the roles of this protein in protecting against cardiac ERS and apoptosis are largely unknown. **Methods:** We investigated the roles of 14-3-3 protein *in vivo* during cardiac ERS and apoptosis induced by pressure overload or thapsigargin injection using transgenic (TG) mice that

showed cardiac-specific expression of dominant negative (DN) 14-3-3 $\eta$ . **Results:** Cardiac positive apoptotic cells and the expression of glucose-regulated protein (GRP)78, inositol-requiring enzyme (Ire)1 $\alpha$ , tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2, CCAAT/enhancer binding protein homology protein (CHOP), caspase-12, and cleaved caspase-12 protein were significantly increased in the pressure-overload induced DN 14-3-3 $\eta$  mice compared with that in the WT mice. Furthermore, thapsigargin injection significantly increased the expression of GRP78 and TRAF2 expression in DN 14-3-3 $\eta$  mice compared with that in the WT mice. **Conclusion:** The enhancement of 14-3-3 protein may provide a novel protective therapy against cardiac ERS and ERS-initiated apoptosis, at least in part, through the regulation of CHOP and caspase-12 via the Ire1 $\alpha$ /TRAF2 pathway.

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## Introduction

The endoplasmic reticulum (ER) is involved in several important functions such as the folding of secretory and membrane proteins. Various conditions including ischemia, hypoxia, exposure to free radicals, elevated protein synthesis, hyperhomocysteinemia, hyperglycemia and gene mutation can cause pathological accumulation of unfolded proteins in the ER - a condition referred to as ER stress (ERS) [1-3]. Excessive and/or prolonged ERS may trigger apoptosis [1-3]. Accumulating evidence has demonstrated that apoptosis initiated by excessive ERS is involved in the pathogenesis of many cardiovascular diseases including diabetic cardiomyopathy [4], transition from cardiac hypertrophy to heart failure [5] and thapsigargin-induced cardiac hypertrophy [5].

The 14-3-3 protein forms a highly conserved family of acidic dimeric proteins with a subunit mass of approximately 30kDa and play important roles in inhibiting apoptosis via the regulation of B-cell lymphoma (Bcl)-2 proteins [6], forkhead transcription factors [7], mitogen-activated protein kinase (MAPK) cascades [8], and apoptosis signal-regulating kinase (Ask)1 [9]. The ability of the 14-3-3 protein to increase the apoptotic threshold of cells has also been validated in dominant negative (DN) 14-3-3 $\eta$  transgenic (TG) mice. In DN 14-3-3 $\eta$  TG mice' heart, DN 14-3-3 $\eta$  represents 50% of the total 14-3-3 protein [8]. We have reported that induction of experimental diabetes in DN 14-3-3 $\eta$  TG mice using STZ exacerbated cardiac apoptosis, at least in part, through the enhancement of c-Jun N terminal kinase (JNK) [10] and Ask1 [11]. Furthermore, DN 14-3-3 $\eta$  TG mice subjected to an established trigger of apoptosis, for example, pressure overload, suffered from massive cardiac apoptosis, at least in part, through the regulation of MAPK cascades [8, 12]. Additionally, the 14-3-3 protein has also been proposed as an ERS response protein and to play a chaperone role as a misfolded protein sweeper [13, 14]. In brain studies, 14-3-3 protein has been reported to play a protective role in ERS. Reduction of the concentration of 14-3-3 $\xi$  to 50% of the control levels activates an ERS response and causes selective apoptosis in the hippocampus [13]. Despite our significant understanding of the physiological role of 14-3-3 protein in inhibiting apoptosis, the roles of these proteins in protecting against ERS and ERS-initiated apoptosis in the diseased heart are largely unknown.

In the present study, we investigated the role of 14-3-3 protein *in vivo* during cardiac ERS and apoptosis

induced by pressure overload or thapsigargin injection using TG mice that showed cardiac-specific expression of DN 14-3-3 $\eta$  and wild type (WT) mice.

## Materials and Methods

### *Generation of DN 14-3-3 $\eta$ TG mice*

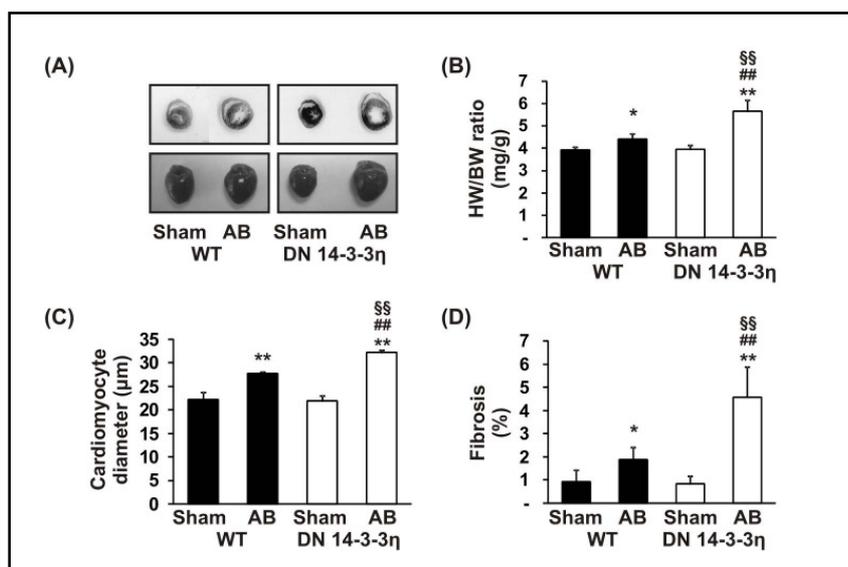
DN 14-3-3 $\eta$  TG mice were generated as described previously [8]. We have reported that in the DN 14-3-3 $\eta$  TG mice heart, DN 14-3-3 $\eta$  represented 50% of the total 14-3-3 protein [8]. Age matched WT C57BL/6 mice (Charles River Japan Inc., Atsugi, Kanagawa, Japan) were used as controls.

### *Ascending aortic banding (AB) surgery*

To induce pressure overload in the mice, we performed ascending AB surgery since this technique provides a more direct and rapid source of pressure overload on the left ventricle (LV) with a significant degree of hypertrophy after 48 hours [15]. Ten- to twelve-week-old male WT mice (WT AB, n=8) and DN 14-3-3 $\eta$  TG mice (DN 14-3-3 $\eta$  AB, n=6) underwent ascending AB surgery using previously described methods [15]. In brief, the mice were anesthetized with Nembutal 50 mg/kg body weight (BW) given intraperitoneally. After an adequate depth of anesthesia had been attained, each mouse was fixed in a supine position with tape. A 5-0 ligature was placed behind the front upper incisors and pulled taut so that the neck was slightly extended. The tongue was retracted and held with forceps, and a 20-G i/v catheter was inserted into the trachea. The catheter was connected to a volume-cycled ventilator supplying supplemental oxygen with a tidal volume of 2.5 ml and a respiratory rate of 120 beats/minutes.

Prior to the incision, the chest was disinfected with povidone iodine solution and 70% ethyl alcohol, and 0.1 ml of 0.1% lidocaine was injected under the skin. The chest cavity was then opened by making an incision in the left second intercostal space. A chest retractor was then applied to facilitate visualization of the surgical field. The pericardial sac was opened and pulled apart, the ascending portion of aorta was dissected from the surrounding tissues, and a 7-0 silk suture was passed underneath the ascending portion of the aorta and ligated using a 26-G needle. The latter was immediately removed to produce a lumen in the stenotic aorta. Then the lungs were overinflated, and the chest cavity, muscles, and skin were closed layer by layer with 6-0 nylon and 6-0 absorbable (for muscles) sutures. The duration of the procedure was about 20-30 minutes. Age and sex matched WT mice (WT sham, n=6) and DN 14-3-3 $\eta$  TG mice (DN 14-3-3 $\eta$  sham, n=4) underwent sham surgery and were used as controls. The sham surgery procedure was identical to that of the ascending AB surgery except that the ascending aorta was not ligated. The mice were then examined after three days. All mice examined were maintained with free access to water and chow throughout the period of study. Animals were treated in strict accordance with the Recommendation from the Declaration of Helsinki and guidelines for animal experimentation of our institute.

**Fig. 1.** Ascending aortic banding (AB)-induced pressure overload resulted in cardiac hypertrophy and fibrosis: (A) Representative photos showing an enlarged heart after the surgery in the WT AB and in the DN 14-3-3 $\eta$  AB mice compared with their shams; (B) A significant increase in the HW/BW ratio was confirmed in the WT AB mice but the effect was more prominent in the DN 14-3-3 $\eta$  AB mice; (C) Quantification of cardiomyocyte diameter; (D) Quantification of myocardial fibrosis. In B, C and D, the black and white bars, respectively, represent the WT and DN 14-3-3 $\eta$  mice. Each bar represents the mean  $\pm$  S.E. (n=4-8 mice). \*p<0.05 and \*\*p<0.01 vs. WT sham, ##p<0.01 vs. DN 14-3-3 $\eta$  sham, §§p<0.01 vs. WT AB.



#### *In vivo thapsigargin injection*

Ten- to twelve-week-old male WT mice (WT Thap, n=6) and DN 14-3-3 $\eta$  TG mice (DN 14-3-3 $\eta$  Thap, n=6) were immunized intraperitoneally with 1 mg/kg BW thapsigargin (Sigma-Aldrich, Inc., St. Louis, MO, USA) using a previously described method [16]. Thapsigargin, an effective inhibitor of Ca<sup>2+</sup> ion pump proteins of the intracellular membranes of the sarcoplasmic reticulum (SR) and the ER of skeletal and cardiac muscles and brain microsomes, has been widely used as an ERS inducer. Age and sex matched WT mice (WT control, n=4) and DN 14-3-3 $\eta$  TG mice (DN 14-3-3 $\eta$  control, n=4) were injected with phosphate buffered saline (PBS) and used as a controls. The mice were examined after 8 hours. All mice examined were maintained with free access to water and chow throughout the period of study. Animals were treated in strict accordance with the Recommendation from the Declaration of Helsinki and guidelines for animal experimentation of our institute.

#### *Ratio of heart weight to body weight (HW/BW)*

At the end of the experiment, the mice were anesthetized with a single intra-peritoneal injection of pentobarbital (50 mg/kg BW), and their hearts were excised. The HW and HW/BW ratio were determined for each mouse. The LV was quickly dissected and cut into two parts. One part was immediately transferred to liquid nitrogen and stored at -80°C for protein analysis. The other part was either stored in 10% formalin and used to make paraffin sections or stored at -80°C after the addition of Tissue-Tek OCT compound (Sakura Finetechnical Co, Ltd, Tokyo) and used to make frozen tissue sections.

#### *Hematoxylin and eosin (HE) staining*

Frozen LV tissues embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co) were cut into 4  $\mu$ m thick sections and fixed in 4% paraformaldehyde (pH 7.4) at room temperature. HE staining was used to assess the cross-sectional area of cardiomyocytes at 400x magnification. All digital photographs were taken using the color image analyzer (CAI-102; Olympus, Tokyo).

#### *Fibrosis analysis*

The area of myocardial fibrosis in Azan-Mallory-stained LV tissue sections was quantified using a color image analyzer (CAI-102; Olympus) by measuring the blue fibrotic areas compared to the red myocardium at 200x magnification. The results were calculated as the ratio of the fibrotic area to the whole area of the myocardium.

#### *Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)*

TUNEL analysis was performed as specified in the *in situ* apoptosis detection kit (Takara Bio Inc., Shiga, Japan). To quantify apoptotic cells, the percentage of TUNEL positive cells was measured at 200x magnification in thirty randomly chosen fields of each three replicates from three samples. The proportion of TUNEL-positive cells was expressed as a percentage of the total cells counted. All digital photographs were taken using the color image analyzer (CAI-102; Olympus).

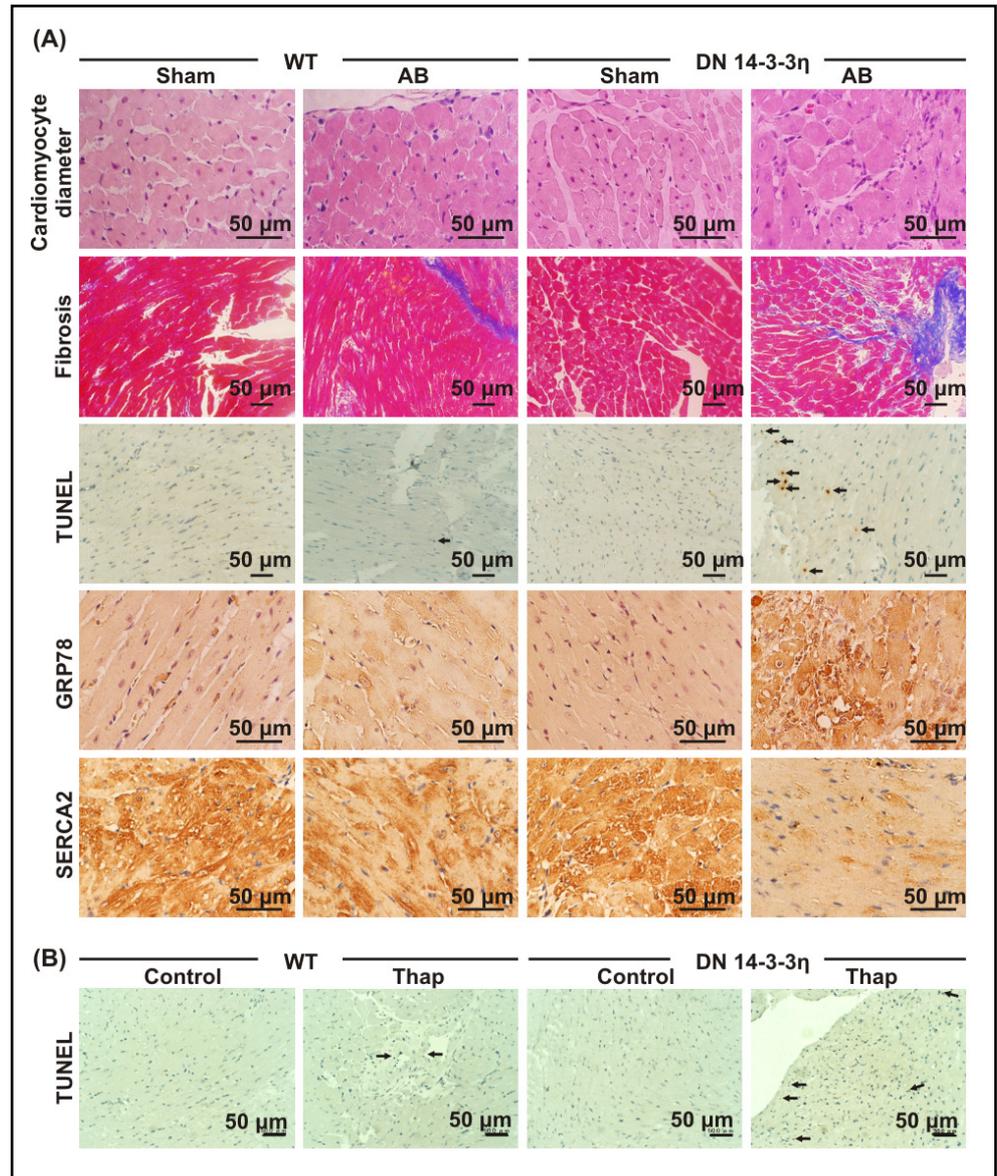
#### *Immunohistochemical staining*

Paraffin embedded sections were used for the detection of glucose-regulated protein (GRP)78 using the goat polyclonal anti-GRP78 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase isoform (SERCA)2 using the goat polyclonal anti-SERCA2 antibody (Santa Cruz Biotechnology, Inc) followed by incubation with the secondary antibody. The GRP78-stained and the SERCA2-stained slides were examined under light microscopy at 400x magnifications. All digital photographs were taken using the color image analyzer (CAI-102; Olympus).

#### *Protein analysis by Western blotting*

Protein lysate was prepared from heart tissue as described previously [10]. The total protein concentrations of the samples were measured by the bicinchoninic acid (BCA) method. For Western blot analysis, 30  $\mu$ g of total protein were loaded and separated by SDS-PAGE (200 V for 40 min), before being electrophoretically transferred to nitrocellulose filters (semi dry

**Fig. 2.** (A) Representative photomicrographs of myocardial tissue sections of aortic-banded mice stained with HE at 400x magnification to evaluate the cardiomyocyte diameter, stained with Azan-Mallory at 200x magnification to evaluate the fibrosis, stained with TUNEL at 200x magnification to evaluate apoptotic cells, stained with anti-GRP78 at 400x magnification to evaluate GRP78 immunoreactivity, stained with anti-SERCA2 at 400x magnification to evaluate SERCA2 immunoreactivity; (B) Representative photomicrographs of myocardial tissue sections of thapsigargin mice stained with TUNEL at 200x magnification to evaluate apoptotic cells.



transfer at 10 V for 30 minute). The filters were then blocked with 5% non-fat dry milk in Tris-buffered saline (20mM Tris (pH 7.6) and 137 mM NaCl) and 0.1% Tween 20, washed, and then incubated with primary antibody. The primary antibodies employed were: goat monoclonal anti-GRP78, mouse polyclonal anti-CCAAT/enhancer binding protein homology protein (CHOP), rabbit polyclonal anti-tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2, rabbit polyclonal anti-inositol-requiring enzyme (Ire)1 $\alpha$ , goat polyclonal anti-atrial natriuretic peptide (ANP) and goat polyclonal anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc); mouse monoclonal anti-galectin-3 (Affinity Bioreagents, Golden, CO, USA); rabbit polyclonal anti-caspase-12 and anti-cleaved caspase-12 (BioVision, Inc., Mountain View, CA, USA) and rabbit polyclonal anti-transforming growth factor (TGF)- $\beta$ 1 (Promega, Madison, WI, USA). After incubation with the primary antibody, the bound antibody was visualized with horseradish peroxidase-coupled secondary antibodies

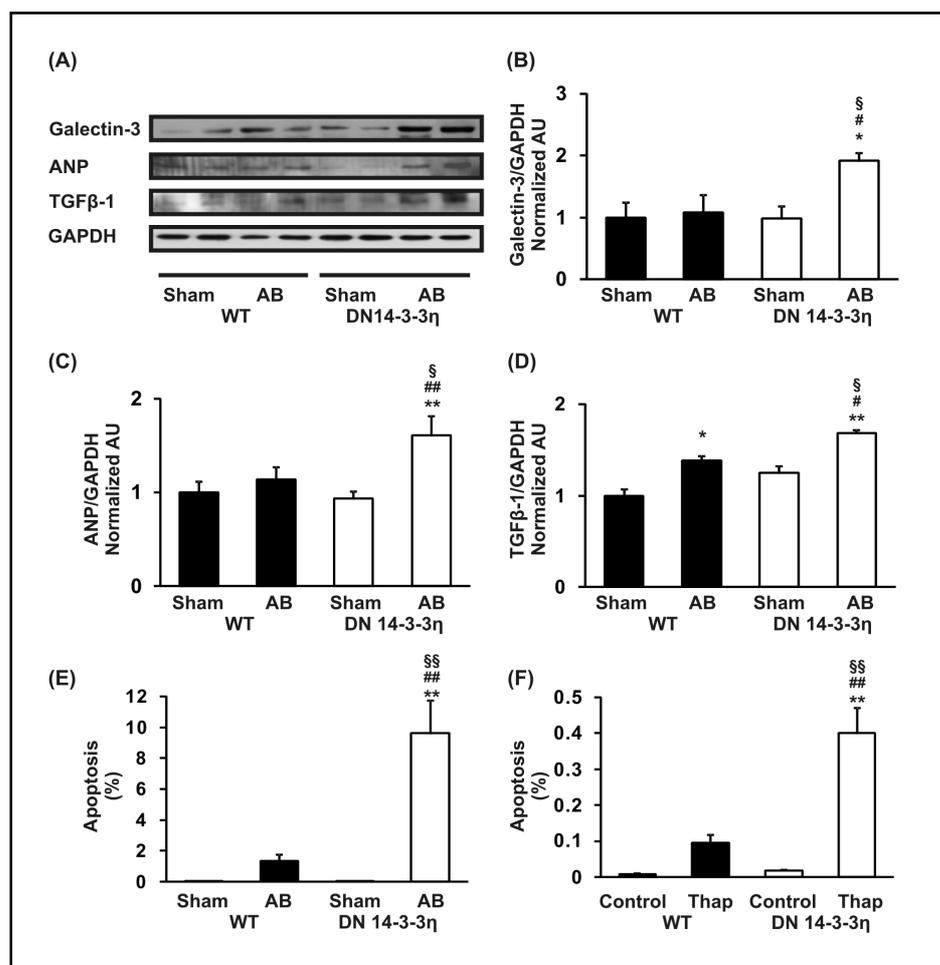
(Santa Cruz Biotechnology, Inc) and chemiluminescence developing agents (Amersham Biosciences, Amersham, Buckinghamshire, UK). The level of expression of each protein in the WT control mice was taken as one arbitrary unit (AU).

For Western blot analysis, all primary antibodies were used at a dilution of 1:1000, and the secondary antibodies were used at a dilution of 1:5000. The films were then scanned using a GT-X700 scanner (Epson, Tokyo, Japan), and band densities were quantified by densitometric analysis using Scion image software (Scion Corporation, Frederick, Maryland, USA).

#### Statistical analysis

Data are presented as means and standard error (SE). Comparison among groups was performed using Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc analysis method, wherever applicable. Differences were considered as statistically significant at probability value < 0.05.

**Fig. 3.** (A) Western immunoblots of cardiac galectin-3, ANP and TGF $\beta$ -1 protein showing two representative experiments out of four. All bands were normalized against GAPDH; (B) Densitometric analysis of the cardiac hypertrophy marker galectin-3 showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice; (C) Densitometric analysis of the cardiac hypertrophy marker ANP showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice; (D) Densitometric analysis of the cardiac fibrosis marker TGF $\beta$ -1 showing a significant increase in its expression in the WT AB mice and in the DN 14-3-3 $\eta$  AB mice; (E) Quantitative analysis of TUNEL staining in aortic-banded mice; (F) Quantitative analysis of TUNEL staining in thapsigargin mice. In B-F, the black and white bars, respectively, represent the WT and DN 14-3-3 $\eta$  mice. Each bar represents the mean  $\pm$  S.E. \* $p$ <0.05 and \*\* $p$ <0.01 vs. WT sham, # $p$ <0.05 and ## $p$ <0.01 vs. DN 14-3-3 $\eta$  sham, § $p$ <0.05 and §§ $p$ <0.01 vs. WT AB.



## Results

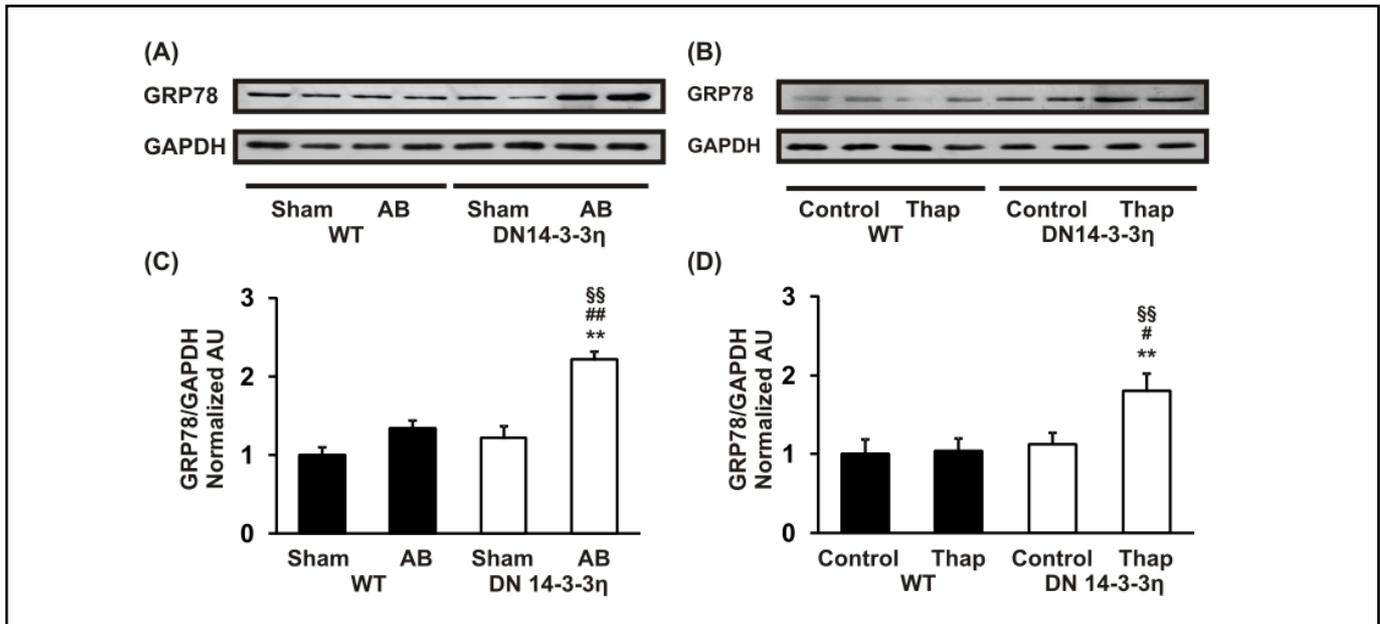
### Pressure overload model

Three days after the ascending AB surgery, prominent cardiac enlargement was observed in the WT AB and the DN 14-3-3 $\eta$  AB mice compared with the sham mice (Fig. 1A). Furthermore, there was a significant increase in the HW/BW ratio in the WT AB ( $p$ <0.05) and the DN 14-3-3 $\eta$  AB mice ( $p$ <0.01) compared with the sham mice (Fig. 1B). The HW/BW ratio was also significantly increased in the DN 14-3-3 $\eta$  AB mice compared with the WT AB mice ( $p$ <0.01). As depicted in Fig. 1C and Fig. 2A, HE staining showed a significant enlargement of cardiomyocyte diameter in the WT AB mice ( $p$ <0.01,  $27.67 \pm 0.38 \mu\text{m}$ ) compared with the WT sham mice ( $22.25 \pm 1.37 \mu\text{m}$ ), and a more prominent increase was seen in the DN 14-3-3 $\eta$  AB mice ( $32.25 \pm 0.46 \mu\text{m}$ ) than in the DN 14-3-3 $\eta$  sham ( $p$ <0.01,  $21.91 \pm 1.07 \mu\text{m}$ ) and in the WT AB mice ( $p$ <0.01). Using Western blot analysis, we further found an increase in cardiac galectin-3 and ANP protein expression in the WT

AB compared with the WT sham mice, however, the increase did not reach significant level in statistics (Fig. 3A-C). Conversely, a significant increase in cardiac galectin-3 and ANP protein expression was observed in the DN 14-3-3 $\eta$  AB mice compared with the DN 14-3-3 $\eta$  sham and the WT AB mice (Fig. 3A-C).

In addition, myocardial fibrosis as one of the results of pressure overload was significantly increased in the WT AB ( $p$ <0.5,  $1.86 \pm 0.53\%$ ) compared with the WT sham mice ( $0.93 \pm 0.47\%$ ), but more significant increase was found in the DN 14-3-3 $\eta$  AB ( $4.56 \pm 1.32\%$ ) than in the DN 14-3-3 $\eta$  sham ( $p$ <0.01,  $0.84 \pm 0.32\%$ ) and in the WT AB mice ( $p$ <0.01, Fig. 1D and Fig. 2A). Further confirmation showed a significant increase in cardiac TGF $\beta$ -1 protein expression in the WT AB compared with the WT sham mice, but more prominent increase was observed in the DN 14-3-3 $\eta$  AB than in the DN 14-3-3 $\eta$  sham and in the WT AB mice (Fig. 3A and D).

Collectively, increases in HW/BW ratio, cardiomyocyte diameter, cardiac galectin-3 and ANP protein expression, myocardial fibrosis and cardiac



**Fig. 4.** GRP78 protein expression during ERS elicited by pressure overload induction or thapsigargin injection: (A) Western immunoblots of cardiac GRP78 showing two representative experiments out of four during ERS elicited by pressure overload induction; (B) Western immunoblots of cardiac GRP78 showing two representative experiments out of four during ERS elicited by thapsigargin injection. In (A) and (B), all bands were normalized against GAPDH; (C) Densitometric analysis of GRP78 showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice; (D) Densitometric analysis of cardiac GRP78 showing a significant increase in its expression in the DN 14-3-3 $\eta$  Thap mice. In C and D, the black and white bars, respectively, represent the WT and DN 14-3-3 $\eta$  mice. Each bar represents the mean  $\pm$  S.E. <sup>\*\*</sup> $p < 0.01$  vs. WT sham/WT control, <sup>#</sup> $p < 0.05$  and <sup>##</sup> $p < 0.01$  vs. DN 14-3-3 $\eta$  sham/DN 14-3-3 $\eta$  control and <sup>§§</sup> $p < 0.01$  vs. WT AB/WT Thap.

TGF $\beta$ -1 protein expression confirmed that three days pressure overload resulted in a significant cardiac hypertrophy in the DN 14-3-3 $\eta$  mice compared with the WT mice.

#### Apoptotic cells analysis

Pressure overload is known to trigger apoptosis in cardiac tissues. As expected, apoptotic cardiac cells were found in significantly greater numbers in the DN 14-3-3 $\eta$  AB mice than in the DN 14-3-3 $\eta$  sham and in the WT AB mice (Fig. 2A and 3E). Conversely, no significant increase in the number of apoptotic cardiac cells was found in the WT AB compared with the WT sham mice (Fig. 2A and 3E).

Consistent with result in pressure overload-induced model, induction of ERS using thapsigargin injection resulted in a significant increase of apoptotic cardiac cells in the DN 14-3-3 $\eta$  Thap compared with the DN 14-3-3 $\eta$  control and the WT Thap mice (Fig. 2B and 3F). Conversely, no significant increase of apoptotic cardiac cells was observed in the WT Thap compared with the WT control mice (Fig. 2B and 3F).

#### Expression of cardiac ERS marker

Pressure overload is known also to induce cardiac ERS [5]. GRP78 is a central regulator of ER function and is widely used as a marker of ERS. In this study, immunohistochemical analysis demonstrated enhanced immunoreactivity of cardiac GRP78 in the DN 14-3-3 $\eta$  AB mice compared with DN 14-3-3 $\eta$  sham and WT AB mice (Fig. 2A). Using Western blot analysis, we further found that three days pressure overload did not significantly induce the expression of the cardiac GRP78 protein in the WT AB mice compared with that in the WT sham mice. On the contrary, cardiac GRP78 protein was significantly induced in the DN 14-3-3 $\eta$  AB mice compared with the DN 14-3-3 $\eta$  sham and the WT AB mice (Fig. 4A and C). Interestingly, during ERS provocation using the ERS inducer thapsigargin, a significant increase in cardiac GRP78 protein expression was not observed in the WT Thap mice compared with the WT control mice but a significant increase was observed in the DN 14-3-3 $\eta$  Thap mice than in the DN 14-3-3 $\eta$  control and in the WT Thap mice (Fig. 4B and D).

**Fig. 5.** Pressure overload resulted in the significant increase of cardiac Ire1 $\alpha$  and TRAF2 protein in the DN 14-3-3 $\eta$  mice: (A) Western immunoblots of cardiac Ire1 $\alpha$  and TRAF2 showing two representative experiments out of four. All bands were normalized against GAPDH; (B) Densitometric analysis of cardiac Ire1 $\alpha$  showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice; (C) Densitometric analysis of cardiac TRAF2 showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice. In B and C, the black and white bars, respectively, represent the WT and DN 14-3-3 $\eta$  mice. Each bar represents the mean  $\pm$  S.E. \*\* $p$ <0.01 vs. WT sham, ## $p$ <0.01 vs. DN 14-3-3 $\eta$  sham, § $p$ <0.05 and §§ $p$ <0.01 vs. WT AB.

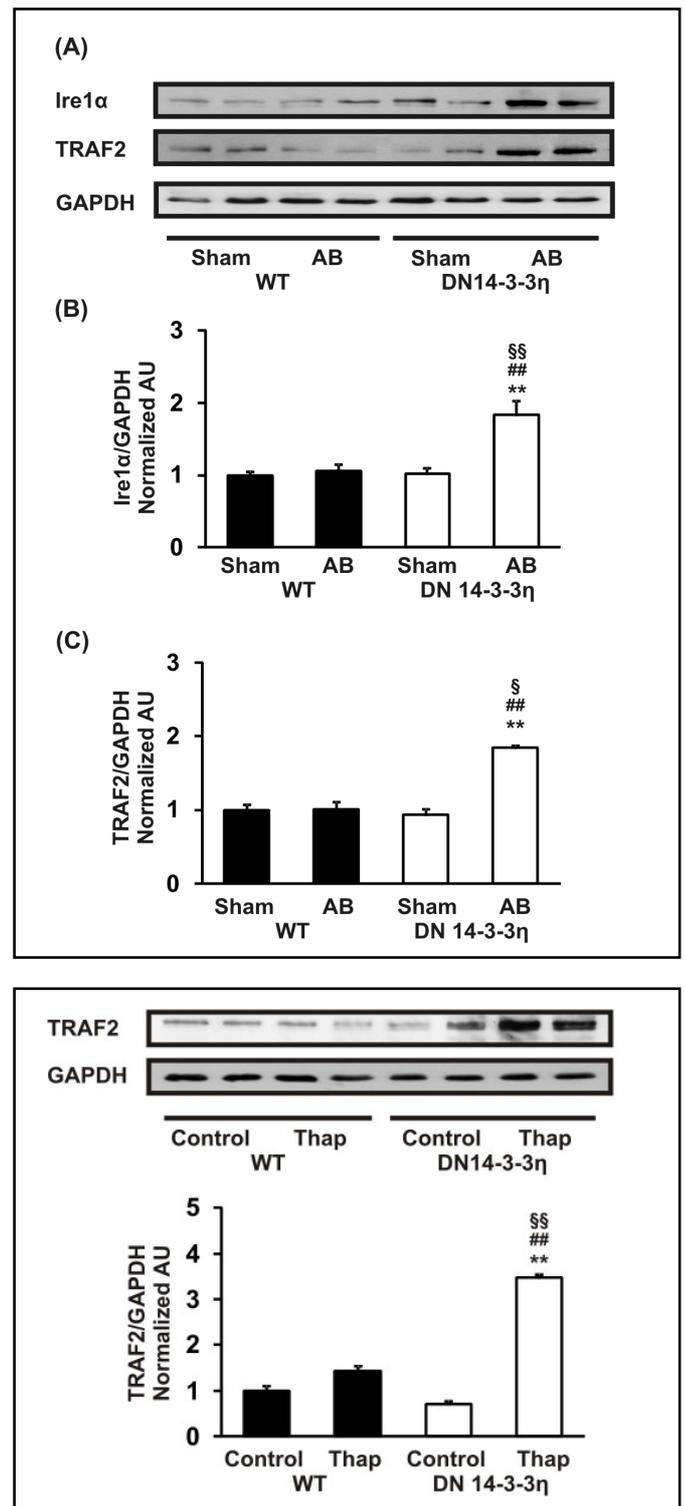
In addition, decreased immunoreactivity of cardiac SERCA2 was observed in the DN 14-3-3 $\eta$  AB mice compared with DN 14-3-3 $\eta$  sham and WT AB mice (Fig. 2A).

#### *Expression of cardiac Ire1 $\alpha$ and TRAF2*

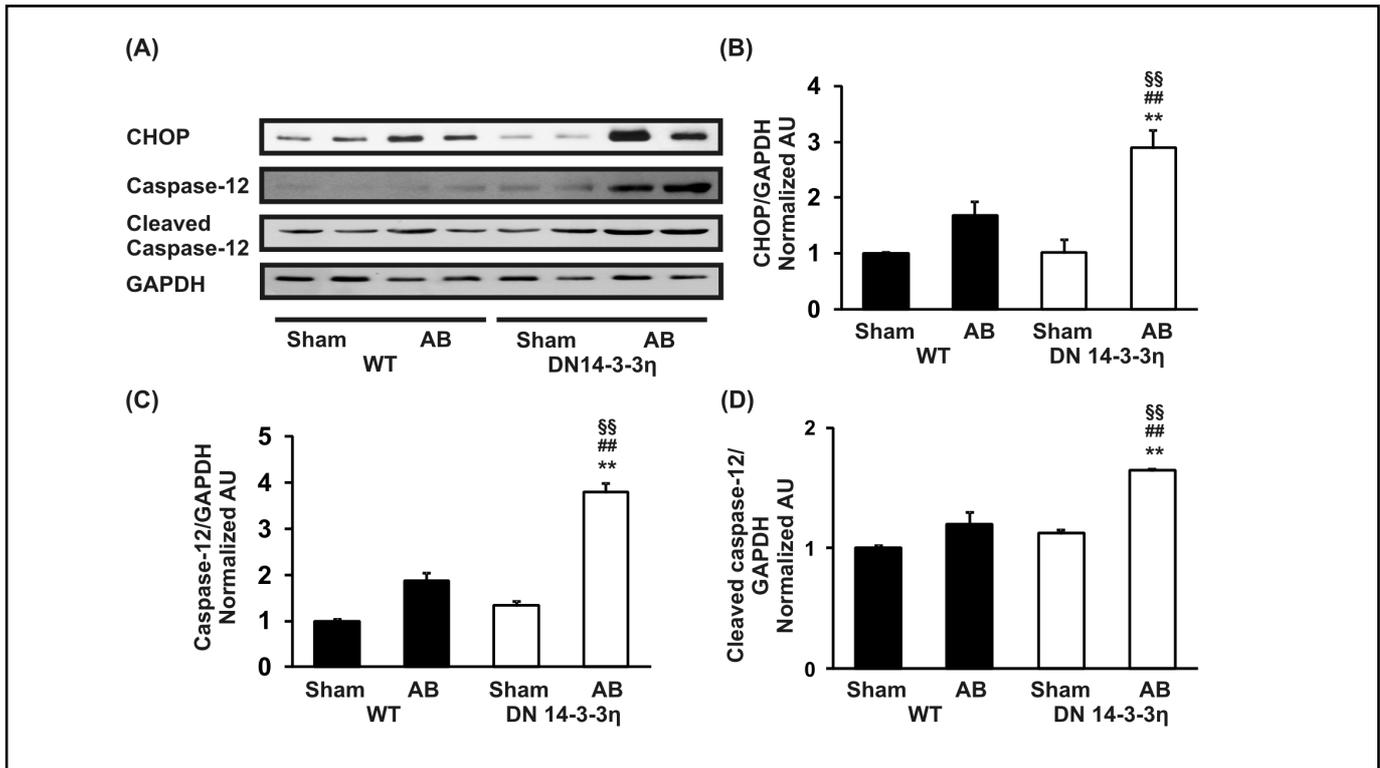
During the accumulation of misfolded proteins, GRP78 is released to aggregate with trans-membrane signaling proteins and launches the unfolded protein response (UPR). We further examined the expression of Ire1 $\alpha$ , a trans-membrane protein. We did not find a significant increase in the cardiac expression of Ire1 $\alpha$  protein in the WT AB mice compared with the WT sham mice (Fig. 5A and B). In contrast, the cardiac expression of Ire1 $\alpha$  protein was significantly increased in the DN 14-3-3 $\eta$  AB mice compared with the DN 14-3-3 $\eta$  sham and WT AB mice (Fig. 5A and B). Additional data showed that the cardiac expression of TRAF2 protein - an adaptor molecule that transduces the ERS signal from Ire1 $\alpha$  to the downstream pathway - was significantly increased in the DN 14-3-3 $\eta$  AB mice compared with the DN 14-3-3 $\eta$  sham and the WT AB mice (Fig. 5A and C). In addition, during ERS provocation with thapsigargin, we found a significant increase in cardiac TRAF2 protein expression only in the DN 14-3-3 $\eta$  Thap mice compared with the DN 14-3-3 $\eta$  control and the WT Thap mice (Fig. 6).

#### *Expression of cardiac CHOP and caspase-12*

High levels of ERS may induce apoptosis through the expression of CHOP. In addition, the Ire1 $\alpha$ /TRAF2/Ask1 complex may also enhance CHOP activity. As depicted in Fig. 7A and B, no significant increase of cardiac CHOP protein expression was observed in the WT AB when compared with the WT sham mice. Conversely, a significant increase in the cardiac CHOP protein expression was observed in the DN 14-3-3 $\eta$  AB mice



**Fig. 6.** TRAF2 protein expression during ERS elicited by thapsigargin (Thap) injection: Western immunoblots of cardiac TRAF2 in two representative experiments out of four. All bands were normalized against GAPDH. Densitometric analysis of cardiac TRAF2 showing a significant increase in its expression in the DN 14-3-3 $\eta$  Thap mice. The black and white bars, respectively, represent the WT and DN 14-3-3 $\eta$  mice. Each bar represents the mean  $\pm$  S.E. \*\* $p$ <0.01 vs. WT control, ## $p$ <0.01 vs. DN 14-3-3 $\eta$  control and §§ $p$ <0.01 vs. WT Thap.



**Fig. 7.** Pressure overload resulted in increased activation of the CHOP and caspase-12 apoptotic pathways in the DN 14-3-3 $\eta$  mice: (A) Western immunoblots of cardiac CHOP, caspase-12, and cleaved caspase-12 showing two representative experiments out of 4. All bands were normalized against GAPDH; (B) Densitometric analysis of cardiac CHOP expression showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice; (C) Densitometric analysis of cardiac caspase-12 expression showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice; (D) Densitometric analysis of cardiac cleaved caspase-12 expression showing a significant increase in its expression in the DN 14-3-3 $\eta$  AB mice. In B, C and D, the black and white bars, respectively, represent the WT and DN 14-3-3 $\eta$  mice. Each bar represents the mean  $\pm$  S.E. \*\* $p$ <0.01 vs. WT sham, # $p$ <0.01 vs. DN 14-3-3 $\eta$  sham and \$\$ $p$ <0.01 vs. WT AB.

compared with the DN 14-3-3 $\eta$  sham and the WT AB mice, consistent with the result of massive apoptosis in the DN 14-3-3 $\eta$  AB mice as previously shown.

Furthermore, the expression of caspase-12 and its cleavage, which are associated with activated Ire1 $\alpha$ , were significantly increased in the DN 14-3-3 $\eta$  AB mice compared with the DN 14-3-3 $\eta$  sham and the WT AB mice (Fig. 7A-D).

## Discussion

Our observations have shown that (1) DN 14-3-3 $\eta$  mice have lower capability than the WT in dealing with three days pressure overload which resulted in a significant increase of cardiac hypertrophy; (2) DN 14-3-3 $\eta$  mice have a lower capability than the WT in dealing with cardiac ERS as shown by the significant elevation of the cardiac

GRP78 protein expression and (3) DN 14-3-3 $\eta$  mice but not WT mice seem to activate Ire1 $\alpha$ /TRAF2 pathway and to activate the ERS-initiated apoptosis pathway, at least in part, through the regulation of CHOP and caspase-12.

### *Role of 14-3-3 protein in cardiac hypertrophy*

We have reported that DN 14-3-3 $\eta$  mice were generated with cardiac-specific expression of DN 14-3-3 $\eta$ . These mice appeared normal at baseline but unable to compensate for pressure-overload induction by transverse aortic constriction (TAC) which resulted in significant increase of death and myocardial apoptosis [8, 12]. We have reported also that exacerbations of cardiac hypertrophy and fibrosis in diabetic DN 14-3-3 $\eta$  mice were significantly and positively correlated with the enhanced expression of protein kinase C (PKC) $\beta$ 2 [17]. In addition, 14-3-3 protein has been reported to inhibit

cardiomyocyte hypertrophy through regulation of the phosphoinositide 3 kinase (PI3K)/PKB/glycogen synthase kinase (GSK)3 $\beta$  and NFAT pathway [18]. Therefore, it was acceptable that cardiac hypertrophy and fibrosis were significantly appeared in the DN 14-3-3 $\eta$  mice compared to the WT mice at three days after pressure overload induction in this study.

#### *Role of 14-3-3 protein in cardiac ERS*

The ER is primarily known as the site of protein folding. Various conditions that perturb cellular energy levels, redox state, and Ca<sup>2+</sup> concentration reduce the protein folding capacity of the ER, resulting in the accumulation of unfolded protein, a condition termed ERS [1-3]. The 14-3-3 protein has been proposed to play important roles in the ER by regulating the forward-trafficking of membrane proteins [19, 20], sweeping misfolded proteins [14], functioning as an ERS response protein [13] and protecting cells against ERS [13].

To observe the role of 14-3-3 protein *in vivo* during cardiac ERS, we induced DN 14-3-3 $\eta$  mice with pressure overload or thapsigargin injection since both pressure overload and thapsigargin are known to induce cardiac ERS [5]. Our findings have shown that three days pressure overload exacerbated cardiac ERS in DN 14-3-3 $\eta$  mice but not in WT mice, as shown by significant elevation of the ERS marker, GRP78. These results demonstrated that the heart of WT mice adapted to increased aortic pressure with hypertrophy and fibrosis but this was not accompanied by a significant induction of ERS. Conversely, significant cardiac hypertrophy and fibrosis appeared in the heart of DN 14-3-3 $\eta$  mice along with significant induction of ERS. These suggest that partial inactivation of cardiac 14-3-3 protein in the DN 14-3-3 $\eta$  mice leads to increased sensibility and vulnerability to ERS under pressure-overload condition. One proposed mechanism is that the highly conserved hydrophobic surface of the amphipathic groove in 14-3-3 protein may allow for the molecular recognition of misfolded proteins in a similar fashion to that by which most conventional molecular chaperones recognize and bind to the hydrophobic surfaces of misfolded proteins [21]. This mechanism may explain why the reduction of 14-3-3 protein expression to 50% of control levels activates an ERS response in the hippocampus [13].

Another possible explanation of mechanism is the involvement of 14-3-3 protein in the regulation of Ca<sup>2+</sup> concentration through SERCA2. In different model of diabetes mellitus, we have reported a decrease in SERCA2 and an increase in GSK3 $\beta$  expression in the

DN 14-3-3 $\eta$  mice compared with WT mice [11, 22], supporting the role of 14-3-3 protein in the regulation of SERCA2 expression at least through the regulation of GSK3 $\beta$  [23]. Additionally, aortic banding-induced pressure overload has been known to alter the cardiac Ca<sup>2+</sup> concentration by reducing the expression of SERCA2 [24]. Consistent with previous studies, our findings have shown that ascending AB surgery decreased SERCA2 immunoreactivity in the DN 14-3-3 $\eta$  mice compared with the WT mice. To test whether 14-3-3 protein involved in the ERS response during cardiac ERS through SERCA2, we further induced ERS using thapsigargin. Thapsigargin has been known to inhibit contraction and Ca<sup>2+</sup> transient in cardiac cells by specific inhibition of the SERCA pump [25], to increase protein levels of ER chaperones in cultured cardiac myocytes [5] and to elicit ERS in time and dose-dependent manner in the neonatal rat cardiomyocytes [26]. In our study, we have shown that the inhibition of SERCA2 using thapsigargin significantly increased cardiac expression of GRP78 protein in the DN14-3-3 $\eta$  mice compared with the WT mice. These suggest that partial inactivation of 14-3-3 protein elicit cardiac ERS at least in part through the regulation of SERCA2 under ERS situations.

Collectively, we have shown *in vivo* that partial inactivation of cardiac 14-3-3 protein in DN 14-3-3 $\eta$  mice during pressure overload or thapsigargin injection will result in a significant increase of cardiac ERS at least in part through regulation of SERCA2, however, further *in vitro* research is required to fully elucidate the role of 14-3-3 protein and cardiac ERS.

#### *Role of 14-3-3 protein in ERS-initiated apoptosis*

The 14-3-3 protein has been reported to play important roles in inhibiting apoptosis via the regulation of Bcl-2 proteins [6], forkhead transcription factors [7], MAPK cascades [8] and Ask-1 [9]. The ability of the 14-3-3 protein to increase the apoptotic threshold of cells has also been validated in DN 14-3-3 $\eta$  TG mice. The induction of experimental diabetes in DN 14-3-3 $\eta$  TG mice has been reported to exacerbate cardiac apoptosis, at least in part, through the regulation of JNK [10] and Ask1 [11]. However, few studies have been published regarding the role of 14-3-3 protein in protecting against ERS-initiated cardiac apoptosis.

It has been proposed that an increase in GRP78 expression would lead to further protective UPR processes through the dissociation of GRP78 from ER trans-membrane proteins including pancreatic ER kinase (PKR)-like ER kinase (PERK), Ire1 $\alpha$ , and activating

transcription factor (ATF)-6 [1-3]. The UPR aims to reduce the accumulation of unfolded proteins and restore normal ER functioning [2]. However, if the stress cannot be resolved and UPR fails to protect against ERS, the signaling switches from pro-survival to pro-apoptotic through Ire1 $\alpha$  [2].

The Ire1 $\alpha$  - a type I trans-membrane protein - seems to be important for the initiation of pro-survival signals in UPR [2], but Ire1 $\alpha$  is also hypothesized to be able to initiate apoptosis [27] and is clearly connected to cell death [28] since active Ire1 $\alpha$  has been shown to recruit the adaptor molecule TRAF2 [29, 30]. Specifically, Ire1 $\alpha$  binds to TRAF2 and causes ERS to activate JNK through its kinase activity [29-31]. Our studies have shown that the cardiac expression levels of both Ire1 $\alpha$  and TRAF2 protein were significantly increased in pressure overload-induced DN 14-3-3 $\eta$  mice along with significant increase of apoptotic cardiac cells. Additionally, significant increases in the apoptotic cardiac cells and cardiac expression of TRAF2 protein were observed in the thapsigargin-induced DN 14-3-3 $\eta$  mice but not in the thapsigargin-induced WT mice, suggesting the possibility of Ire1 $\alpha$ /TRAF2 pathway activation during ERS in DN 14-3-3 $\eta$  mice which leads to activation of apoptosis. It should be noted that activation of JNK by Ire1 $\alpha$ /TRAF2 requires the presence of Ask1 so it has therefore been proposed that ERS induces the formation of Ire1 $\alpha$ /TRAF2 complex, which in turn leads to Ask1/JNK activation [31]. We have reported that Ask1 were significantly enhanced in DN 14-3-3 $\eta$  mice at three days after STZ injection. The enhanced Ask1 activity observed in DN 14-3-3 $\eta$  mice has been reported to associate with dephosphorylation of phospho-Ask1 (Ser-967), which has been identified as the 14-3-3 protein-binding site for Ask1 [11]. It seems during pressure overload and thapsigargin injection, partial inactivation of 14-3-3 protein will result in the failure of the UPR to protect cells from apoptosis. These result appeared at least through the enhanced Ask1 which may contribute to the activation of Ire1 $\alpha$ /TRAF2 pathway.

When ERS is excessive and/or prolonged, however, the initiation of apoptosis is promoted by transcriptional induction of CHOP, the caspase-12 dependent pathway, or JNK [32, 33]. Overexpression of CHOP is also known to induce apoptosis by increasing Bim (a pro-apoptotic member of the Bcl-2 family) expression [34] and

suppressing the expression of Bcl-2 [35]. We have shown that the cardiac expression of CHOP was significantly increased along with that of caspase-12 and its cleavage in the pressure overload-induced DN 14-3-3 $\eta$  mice compared with the WT mice. These findings may be related with the previous finding that the Ire1 $\alpha$ /TRAF2 pathway may have been activated in the pressure overload and in the thapsigargin-induced DN 14-3-3 $\eta$  mice. The Ire1 $\alpha$ /TRAF2 pathway has been known to enhance CHOP activity at the posttranscriptional level [36]. TRAF2 has also been demonstrated to interact with procaspase-12 and promotes its cleavage by transducing signals from activated Ire1 $\alpha$  [37]. Moreover, it has been reported that Ask1 is activated within 15 min in response to ERS and that caspase-12 activation occurs much slower after an increase in the production of inactive caspase-12 precursor [38, 39]. Thus, Ire1 $\alpha$ , TRAF2, and Ask1 may lie upstream of CHOP and caspase-12.

## Conclusion

Our findings suggest that in diseased condition partial inactivation of cardiac 14-3-3 protein in DN 14-3-3 $\eta$  mice exacerbated cardiac ERS and activated the ERS-initiated apoptosis pathway, at least in part, through the regulation of the CHOP and caspase-12 via Ire1 $\alpha$ /TRAF2 pathways. These results support the assertion that the enhancement of 14-3-3 protein may provide a novel protective therapy against cardiac ERS and ERS-initiated apoptosis. However, further studies will be required to fully elucidate whether stabilization and increased expression of 14-3-3 $\eta$  protein protects from cardiac ERS and ERS-initiated apoptosis.

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