RESEARCH ARTICLES

POTENTIAL PROTECTIVE ROLE OF 14-3-3 PROTEIN IN PATHOLOGICAL CARDIAC HYPERTROPHY THROUGH THE REGULATION OF ENDOPLASMIC RETICULUM STRESS: ROLE OF CALRETICULIN

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ABSTRACT

Background: 14-3-3 protein plays an important role in protecting cardiac cells from hypertrophy and endoplasmic reticulum (ER) stress during pressure overload elicited by aortic banding (AB) surgery; however, the relation among these protective roles is largely unknown.

Methods: We investigated the *in vivo* role of 14-3-3 protein in two protocols involving C57/BL-6 mice and dominant negative (DN) 14-3-3η mice subjected to three- or seven-days pressure overload stimulation by applying AB surgery. The protein expressions of cardiac hypertrophy and ER stress markers, including atrial natriuretic peptide (ANP), galectin-3, glucose-regulated protein (GRP)78, calreticulin as well as 14-3-3 protein was analyzed by western blot.

Results: Three- or seven-day pressure overload stimulation significantly increased the heart weight/body weight (HW/BW) ratio along with the increment of cardiomyocyte diameter. Protein concentration of ANP, GRP78 as well as 14-3-3 increased significantly in the aortic-banded C57/BL-6 mice compared to the sham mice. Partial depletion of 14-3-3 protein significantly increased the protein expression of ANP, Galectin-3, GRP78, and calreticulin in three- or seven-days aortic-banded DN 14-3-3η mice

Conclusion: These results suggest that 14-3-3 protein, as a molecular chaperone, protects against pathological cardiac hypertrophy, at least in part, by maintaining the normal ER function through the regulation of GRP78 and calreticulin.

Keywords: 14-3-3, cardiac hypertrophy, endoplasmic reticulum stress, GRP78, calreticulin

INTRODUCTION

During physiological or pathological stimuli, the heart may change from normal to hypertrophic as an adaptive response to overcome the stimuli.[1] Some pathological responses, including abnormal protein synthesis, and Ca^{2+} depletion or overload are involved in the development of cardiac hypertrophy.[2] In hypertrophied cardiac myocytes, intracellular Ca^{2+}, which is predominantly regulated by the endoplasmic reticulum (ER), is reported to be significantly elevated.[3] Furthermore, the prolongation of ER stress in pressure-overloaded mice play important roles in the transition from cardiac hypertrophy to heart failure.[4] These studies have demonstrated that normal ER function is essentially required to maintain the normal growth of cardiomyocytes. Recently, evidence have shown that the induction of ER dysfunction with thapsigargin in neonatal cardiomyocytes resulted in a significant cardiac hypertrophy through the regulation of the calcineurin and myocyte enhancer factor (MEF)-2c pathways in a time and dose-dependent manner.[5] Therefore, in cardiac hypertrophy, it is intriguing to speculate that hypertrophic stimuli activate ER stress, and that this ER stress subsequently amplifies the hypertrophic process.

The 14-3-3 protein, a molecular chaperone, essentially contributes in maintaining multiple cellular processes of the normal cell including coordination of cell adhesion and motility, the prevention of apoptosis, cell cycle checkpoints and DNA repair, differentiation and senescence, cell trafficking and the onset of cell.[6,7] Additionally, this protein subsequently inhibits cardiomyocyte hypertrophy by regulating the phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB–also called Akt)-nuclear factor of activated T-cells (NFAT) pathway.[8] Partial depletion of 14-3-3 protein *in vivo* in diabetes mellitus and a swimming stress model elicited pathological cardiac hypertrophy through the

regulation of many pathways including NFAT, glycogen synthase kinase (GSK)3β,[9] and p38 mitogen-activated protein kinase (MAPK).[10] We have also reported that ascending aortic banding (AB) surgery induced significant pathological cardiac hypertrophy as well as ER disturbances, as recognized by the up-regulation of glucose-regulated protein (GRP)78 protein expression, in dominant negative (DN) 14-3-3η mice.[11] Thus, we hypothesized that 14-3-3 protein protects the transition from normal to hypertrophic heart, at least in part, by maintaining a normal ER function.

This study is an extension of our previous study in which the partial inactivation of 14-3-3 protein during pressure overload elicited pathological cardiac hypertrophy processes along with ER stress activation. However, in our previous study, we did not ascertain the connecting role of 14-3-3 protein in hypertrophic growth of the heart and ER stress.

METHODS

Generation of DN 14-3-3η mice

The generation of DN 14-3-3η mice were done as described in previously published paper.[12] In brief, DN 14-3-3η represented 50% of the total 14-3-3 protein in the DN 14-3-3η mice heart.[12] Wild-type (WT) C57BL/6 mice (Charles River Japan Inc., Atsugi, Kanagawa, Japan) were used as control.

Ascending AB surgery

Ascending AB surgery were applied to create pressure overload since this technique provides a significant degree of hypertrophy after 48 hours.[13] Ascending AB surgery were done in two protocols. First protocol, ten- to twelve-week-old male C57/BL-6 mice were subjected to three- or seven days ascending AB surgery with sham mice as negative control. Second protocol, three- or seven days ascending AB surgery were done in the DN 14-3-3η mice and their WT using previously described methods.[11,13] The sham group of all protocols underwent identical surgery to that of the AB surgery except that the ascending aorta was not ligated. During the period of the study, all mice received free access to water and chow without any food restriction. All protocols were done in strict accordance to the guidelines for animal experimentation as stated in the previous published work.[11]

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

After three- or seven-days surgery, the mice from two protocols were injected with pentobarbital (50 mg/kg BW). Hearts were excised and weighed, then HW/BW ratio were analyzed for each mouse. The LV of the mouse was dissected into two parts. First part went to liquid nitrogen for protein analysis. The other part was stored at -80ºC to make frozen tissue sections.

Hematoxylin and eosin (HE) staining

LV tissues were sliced into 4 µm thick and fixed in 4% paraformaldehyde (pH 7.4). HE staining method was applied to evaluate the cardiomyocytes diameter under the magnification of 400x. All photographs were digitally taken and analyzed by the image analyzer application (CAI-102; Olympus, Tokyo).

Protein analysis by Western blotting

Heart tissues were homogenized to be used as protein lysate as described in the previous publication.[9] Bicinchoninic acid (BCA) method were applied to estimate the total protein concentrations of the samples. Thirty µg of total protein were used to Western blot analysis by loading and separating the total protein in the SDS-PAGE. After being transferred to nitrocellulose filters, the filters were then blocked with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween 20. For the incubation with primary antibody, the filters were then washed and blocked with antibody. The primary antibodies applied for western blot were goat polyclonal anti-atrial natriuretic peptide (ANP), goat monoclonal anti-GRP78, goat polyclonal anti-calregulin (also called calreticulin), and goat polyclonal anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc); mouse monoclonal anti-galectin-3 (Affinity Bioreagents, Golden, CO, USA). To visualize the protein band, the bound antibody was incubated with secondary antibodies (Santa Cruz Biotechnology, Inc) and chemiluminescence agents (Amersham Biosciences, Amersham, Buckinghamshire, UK). All primary antibodies were diluted at 1:1000 while the secondary antibodies were diluted at 1:5000. Al films were scanned by GT-X700 scanner (Epson, Tokyo, Japan). Band density analysis was quantified Scion image software (Scion Corporation, Frederick, Maryland, USA).

Statistical analysis

All quantitative data were estimated as means with standard error (SE). Comparison among groups was performed using Student's t-test or one-way analysis of variance (ANOVA), wherever applicable. Statistically significant were defined as probability value < 0.05.

RESULTS

FIRST PROTOCOL

Pathological cardiac hypertrophy animal model

As depicted in the Table 1, three- or seven-days ascending AB surgery elicited significant increases of the HW/BW ratio in the aortic-banded C57/BL-6 mice ($p<0.01$). We confirmed this enlargement by measuring cardiomyocyte diameter in HE-stained slides under 400x magnifications. A significant increase of cardiomyocyte diameter was observed in the HE-stained slides of the ABD3 and ABD7 mice compared to the sham mice (Figure 1).

Table 1. HW/BW ratio among groups in the first protocol

Parameter	Sham	ABD3	ABD7
HW/BW ratio	3.94±0.26	4.41±0.22**	5.54±0.66**

HW = heart weight; BW = body weight; ABD3 = aortic banding surgery 3 days; ABD7 = aortic banding surgery 7 days
**p<0.01 compared to the sham group

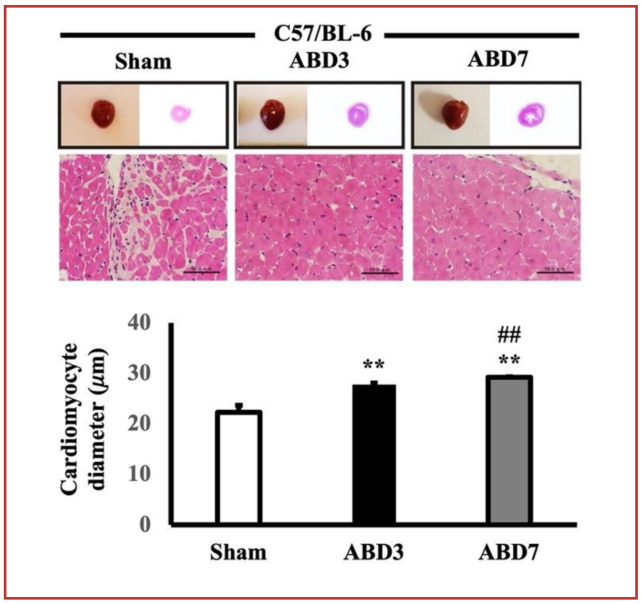

Figure 1. First protocol: three- or seven-days pressure overload stimulation elicited pathological cardiac hypertrophy in C57/BL-6 mice as proven by the significant increase of cardiomyocyte diameter. Sham = mice without ligation in ascending aorta; ABD3 = mice with three days ascending aortic banding (AB); ABD7= mice with seven days ascending AB. **p<0.01 vs. sham; ##p<0.01 vs. ABD3.

Cardiac hypertrophy and ER stress markers

Protein related to pathological cardiac hypertrophy including ANP was significantly upregulated in the ABD3 and ABD7 group mice when compared to the non-aortic banded mice (Figure 2). Consistent with the hypertrophic marker, GRP78, was significantly increased in the ABD3 and ABD7 mice compared to the sham mice (p<0.05). Overall, 14-3-3 protein was upregulated as well as the hypertrophic and the ER stress markers. Collectively, during transition from normal to hypertrophic condition, ER stress as well as molecular chaperone 14-3-3 were activated.

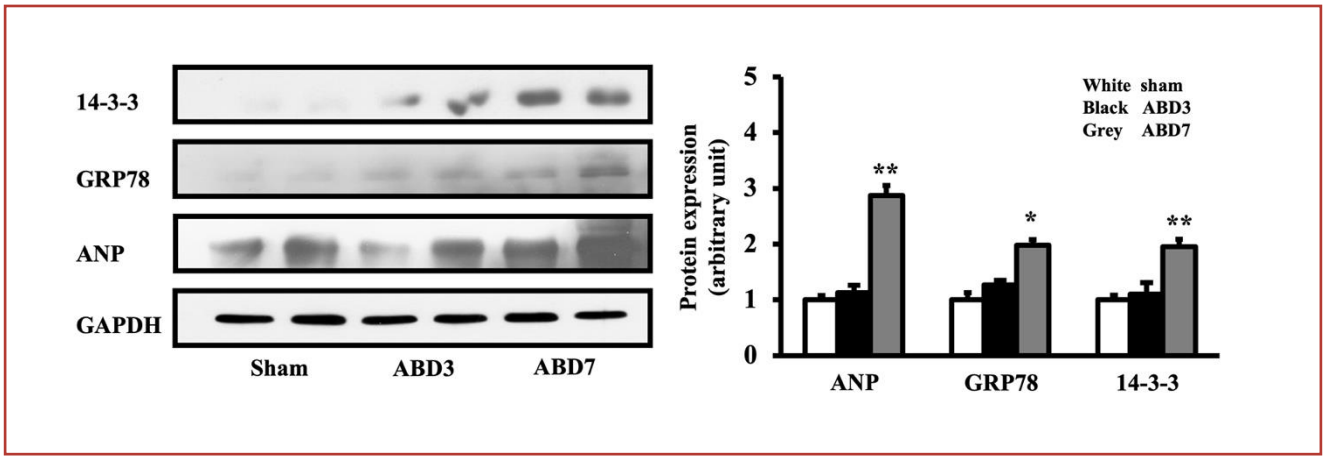

Figure 2. First protocol. Left panel: western blot bands of cardiac ANP, GRP78, and 14-3-3 proteins showing two representative experiment. Right panel: quantitative analysis of cardiac ANP, GRP78, and 14-3-3 proteins. The white, black, and grey bars, represent the sham, ABD3 and ABD7 of C57/BL-6 mice, respectively. All bands were quantified against GAPDH; ABD3 = mice with three days ascending aortic banding (AB); ABD7= mice with three days ascending AB. *p<0.05 and **p<0.01 vs. sham

SECOND PROTOCOL

Pathological cardiac hypertrophy animal model

To investigate the potential role of 14-3-3 protein during the ER stress condition elicited by the hypertrophic stimuli, we generated DN 14-3-3η mice as previously described.[12] As depicted in Table 2, three- and seven-days ascending AB surgery elicited significant increases of HW/BW ratio in the pressure-overloaded WT and DN 14-3-3η mice when compared to their shams. However, more significant increases of HW/BW ratio were observed in the aortic-banded DN 14-3-3η mice compared to aortic-banded WT mice.

Table 2. HW/BW ratio among wild-type (WT) and DN 14-3-3η mice

Parameter	Sham	ABD3	ABD7
WT mice	3.94±0.25	4.40±0.21**	5.43±0.65**
DN 14-3-3η mice	3.83±0.21	5.12±0.61**#	6.31±0.41**#

HW = heart weight; BW = body weight; ABD3 = aortic banding surgery three days; ABD7 = aortic banding surgery seven days; WT = wild-type; DN 14-3-3η = dominant-negative 14-3-3η mice. **p<0.01 compared to the sham WT or sham DN 14-3-3η mice; #p<0.05 compared to WT ABD3 or WT ABD7 mice

Expression of hypertrophic and ER stress markers in pressure overloaded DN 14-3-3η mice

Hypertrophic stimulation was defined with the significant upregulation of Galectin-3 in both aortic banded WT and the DN 14-3-3η mice. More prominent hypertrophy was found in the aortic-banded DN 14-3-3η mice compared to the WT mice. Subsequently, we have reported that the expression levels of ER stress markers were upregulated in the pressure overload DN 14-3-3η mice three days after the surgery.[11] However, it has not been evaluated whether ER stress plays a pivotal role in the development of pathological cardiac hypertrophy. In the aortic-banded sham WT mice, significant ER stress, as recognized by GRP78, was observed at seven days after surgery. In addition, GRP78 was also significantly increased in the three- or seven-days pressure overloaded DN 14-3-3η mice compared with those observed in the WT ABD3 ABD7 mice (Figure 3). These results showed that earlier activation of ER stress were observed in the transgenic mice under the same degree of hypertrophic stimulation. Thus, it is hypothesized that 14-3-3 protein is required to maintain normal ER function during pathological stimulation such as pressure overload. Consistent with these results, protein expression of calreticulin were significantly found in the aortic banded WT mice compared to the sham mice. However, more calreticulin was found in the pressure overloaded DN 14-3-3η mice. Therefore, calreticulin and 14-3-3 protein played important roles as connecting factor in the pathological cardiac hypertrophy.

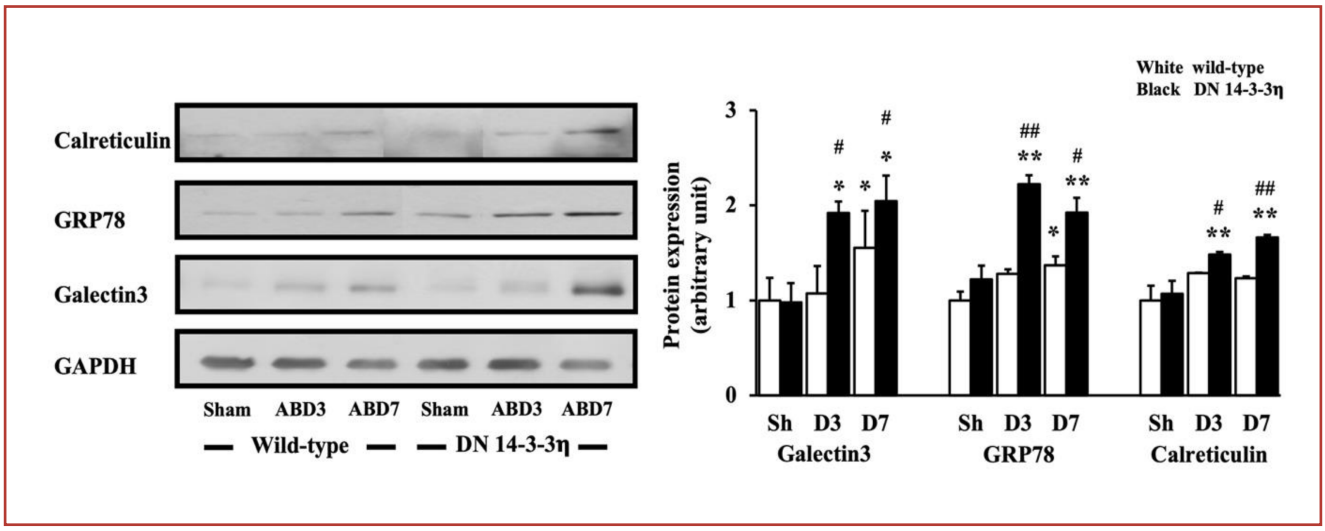

Figure 3. Second protocol. Left panel: western blot bands of cardiac galectin-3, GRP78, and calreticulin proteins showing one representative experiment. Right panel: quantitative analysis of cardiac galectin-3, GRP78, and calreticulin proteins. All bands were quantified against GAPDH. The black and white bars, represent the WT and DN 14-3-3η mice, respectively.

WT = wild-type; DN = dominant negative; ABD3 = mice with three days ascending aortic banding (AB); ABD7= mice with seven days ascending AB.

*p<0.05 and **p<0.01 vs. WT or DN 14-3-3η sham; #p<0.05 and ##p<0.01 vs. WT ABD3 or WT ABD7.

DISCUSSION

Our observations have shown that (1) more significant degree of pathological cardiac hypertrophy as well as a cardiac ER stress response were observed in the DN 14-3-3η mice than in the WT mice suggesting the involvement of 14-3-3 protein in the transition from normal to cardiac hypertrophy; (2) Partial inactivation of 14-3-3 protein elicited more calreticulin which is known to play role in both of cardiac hypertrophy and ER stress; (3) 14-3-3 contributes to the prevention of cardiac hypertrophy development at least in part through the calreticulin pathway.

In hypertrophied cardiac myocytes, intracellular Ca^{2+}, which is predominantly regulated by the ER, is reported to be significantly elevated.[3] Additionally, recent evidences have shown that prolonged ER stress essentially contributes to the transition from normal heart to hypertrophic one,[4] and the induction of ER dysfunction with thapsigargin in neonatal cardiomyocytes resulted in significant cardiac hypertrophy in a time and dose dependent manner.[5] Through these studies, it was found that normal ER function is required to maintain the normal cardiomyocyte growth.

The 14-3-3 protein, a 30kDa molecule, is a molecular chaperone that forms a family of acidic dimeric proteins.[6,7] We have previously found in vivo that during pressure overload stimulation, partial depletion of 14-3-3 protein resulted in a higher degree of pathological cardiac hypertrophy, as well as a higher extent of cardiac ER stress response activation.[11] However, how 14-3-3 protects against cardiac hypertrophy through the regulation of ER stress remains elusive. In this recent report, we have shown that the duration of pressure overload stimulation affects not only the development of pathological cardiac hypertrophy but also the activation of the myocardial expression of GRP78 and calreticulin, which are ER stress markers. Nevertheless, the higher degree of pathological cardiac hypertrophy, GRP78 and calreticulin response activation during partial depletion of 14-3-3 protein have shown us that 14-3-3 protein plays important role in the prevention of ER disturbances. These ER disturbances are considered to have subsequently enhanced the hypertrophic processes. In accordance with our results, in obese and insulin-resistant individuals, ER stress and the expression of 14-3-3 protein were significantly elevated, although the mechanism responsible for these effects has not been fully elucidated.[14] Highly conserved hydrophobic surface of the amphipathic groove in the 14-3-3 protein probably helps the molecular recognition of misfolded proteins.[15] Furthermore, 14-3-3 protein has also been shown to maintain normal ER function by regulating the forward-trafficking of membrane proteins[16,17] and to act as a stress-induced molecular chaperone that dissolves and re-naturalizes thermally aggregated proteins.[18] Partial inactivation of 14-3-3ζ up to 50% of the normal levels elicits apoptosis in the hippocampus through the ER stress pathway.[19] Through these studies, it is considered that the partial inactivation of 14-3-3 during hypertrophic stimulation results in increased misfolded protein levels, which further activates the ER stress; however, this explanation should be confirmed in in vitro studies.

Additionally, a Ca^{2+} binding chaperone of the ER and one of 14-3-3 interactors, calreticulin, has been reported to work as an upstream of calcineurin and MEF-2c pathway through a signal transduction cascade that connects the ER and nucleus during cardiac development.[21,22] Therefore, calreticulin is an important target of MEF-2, and increased expression of calreticulin enhances MEF-2 transcriptional activity.[21,22] We have shown that the protein expression levels of calreticulin in the DN 14-3-3η mice were significantly increased on day 3 and 7 after surgery. Thus, during partial inactivation of 14-3-3 protein, it seems that calreticulin was the connecting factors between ER disturbance and the activation of cardiac hypertrophy. Therefore, we conclude that, through its molecular chaperone function to recognize misfolded protein, 14-3-3 protein play a pivotal role in the prevention of pathological cardiac hypertrophy, at least in part, by maintaining normal ER function through the regulation of the calreticulin-dependent MEF-2c pathway.

CONCLUSION

These results suggest that 14-3-3 protein, as a molecular chaperone, protects against pathological cardiac hypertrophy, at least in part, by maintaining the normal ER function through the regulation of GRP78 and calreticulin.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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