Attenuation of CHOP-mediated Myocardial Apoptosis in Pressure-overloaded Dominant Negative p38 α Mitogen-activated Protein Kinase Mice

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Key Words

p38 MAPK • Endoplasmic reticulum stress • Apoptosis • Pressure overload

Abstract

Background/Aims: Pressure overload stimulation is known to elicit disturbances in the endoplasmic reticulum (ER), which leads to ER stress (ERS). p38 mitogen-activated protein kinase (MAPK) plays an important role in mediating apoptotic processes, however, the roles of this kinase in activating ERS-initiated apoptosis in pressure-overloaded hearts are largely unknown. **Methods:** We clarified the role of p38 α MAPK in ERS-associated apoptosis by subjecting transgenic mice displaying cardiac specific dominant negative (DN) mutant p38 α MAPK over-expression to seven day pressure overload. **Results:** Seven days pressure overload resulted in the same extent of cardiac hypertrophy and ERS in the wild-type (WT) and DN p38 α mice compared with the sham mice. It also activated inositol-requiring enzyme (Ire)-

1 α and its downstream molecule, tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2 in the WT and DN p38 α mice compared with the sham mice. Interestingly, increased myocardial apoptosis and the up-regulation of CCAAT/enhancer binding protein homology protein (CHOP) expression compared with those in the sham mice were found in the aortic-banded WT mice, but not in the DN p38 α mice. **Conclusion:** Partial inhibition of p38 α protein blocked the activation of CHOP-mediated apoptotic processes during pressure overload by partially inhibiting signaling from the Ire-1 α /TRAF2 to its downstream molecule, CHOP.

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Introduction

The endoplasmic reticulum (ER) plays essential roles in multiple cellular processes that are required for cell survival and normal cell functions such as the folding of

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secretory and membrane proteins [1, 2]. Multiple disturbances including ischemia, hypoxia, exposure to free radicals, elevated protein synthesis, hyperhomocysteinemia, hyperglycemia and gene mutation can cause the pathological accumulation of unfolded proteins in the ER – a condition referred to as ER stress (ERS), which subsequently triggers an evolutionarily conserved response termed the unfolded protein response (UPR) [1-3]. The UPR aims to reduce the accumulation of unfolded proteins and restore normal functions; however, if the stress persists and the UPR fails to protect the cells against ERS, the signaling switches from pro-survival to pro-apoptotic [2].

p38 mitogen-activated protein kinase (MAPK) is a member of the MAPK family that is activated by physical and chemical stressors resulting in growth promotion, apoptosis, oxidative stress, and vasoconstriction [4, 5]. It contains α , β , γ , and δ isoforms, the α isoform of which is the major isoform in the human heart [6]. Accumulating evidence has shown that p38 MAPK plays an important role in mediating apoptotic processes in many stress-induced cells [7-9]. Recently, p38 MAPK has been reported to be involved in ERS-induced cell death and autophagy in human gingival fibroblasts [10]. This mechanism may be mediated by the ability of p38 MAPK to phosphorylate and activate the ERS-initiated transcription factor for apoptosis, CCAAT/enhancer binding protein homology protein (CHOP), through inositol-requiring enzyme (Ire)-1 [11]. Additionally, upon disruption of ER calcium homeostasis, another ER transmembrane, protein kinase-like ER kinase (PERK), is required to activate the stress-activated MAPKs [12, 13]. These studies suggest that ER disturbances activate apoptotic processes in cells at least in part through p38 MAPK.

Despite our significant understanding of the physiological role of p38 MAPK in apoptosis activation, the roles of this kinase in mediating ERS-associated apoptosis in models of cardiac hypertrophy are largely unknown. In the present study, to clarify the role of p38 α MAPK in ERS-associated apoptosis, we subjected transgenic mice that displayed cardiac specific dominant negative (DN) mutant p38 α MAPK over-expression to seven days pressure overload.

Materials and Methods

Generation of DN p38 α MAPK mice

Transgenic DN p38 α MAPK mice in Swiss Black genetic background were generated as described previously [14] at

the Neuroscience Transgenic Facility of Washington University School of Medicine. Progeny was analyzed by polymerase chain reactions to detect transgene integration using mouse-tail DNA as template. DN p38 α MAPK mice were compared to wild type (WT) littermates in every experiment.

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, 16]. Ten- to twelve-week-old male WT (WT AB; n = 6) and DN p38 α MAPK mice (DN p38 α AB; n = 7) were anesthetized intraperitoneally with Nembutal 50 mg/kg body weight. After an adequate depth of anesthesia had been attained, 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 20-G i/v catheter was inserted into the trachea and 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. The chest cavity was opened by making an incision in the left second intercostal space. A chest retractor was applied to visualize the surgical field. The pericardial sac was opened, the ascending portion of aorta was dissected, and a 7-0 silk suture was passed underneath the ascending portion of the aorta and ligated using 26-G needle. The latter was immediately removed to produce a lumen in the stenotic aorta. The chest cavity, muscles, and skin were closed layer by layer with 6-0 nylon and 6-0 absorbable sutures. Sham mice (WT sham = 8 mice; DN p38 α sham = 5 mice) underwent procedure that identical to that of the AB surgery except that the ascending aorta was not ligated. All mice examined were maintained with free access to water and chow until seven days after surgery. 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 (HW) to body weight (HW/BW)

Seven days after AB surgery, 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 μ m thick sections and fixed in 4% paraformaldehyde (pH 7.4) at room temperature. HE staining was used to assess the cardiomyocyte diameter at 400x magnification. All digital photographs were taken using the color image analyzer (CAI-102; Olympus, Tokyo).

Fig. 1. (A) Western immunoblots of cardiac phospho-p38 protein (n=4 mice) normalized against p38, phospho-MAPKAPK-2 protein (n=4 mice) normalized against MAPKAPK-2 and p90RSK protein (n=4 mice) normalized against RSK 1/2/3; (B) Densitometric analysis of the cardiac phospho-p38 expression; (C) Densitometric analysis of the cardiac phospho-MAPKAPK-2 expression and (D) Densitometric analysis of the cardiac p90RSK expression. The black and white bars, respectively, represent the WT and DN p38 α mice. Each bar represents the mean $\pm$ S.E. *p<0.05 and **p<0.01 vs. WT or DN p38 α sham, #p<0.05 and ##p<0.01 vs. WT AB.

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 100x 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) assay

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 100x 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) followed by incubation with the secondary antibody. The GRP78-stained 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 [17]. The total protein concentrations of the samples were measured by the bicinchoninic acid (BCA) method. For

Western blot analysis, 50 μ g of total protein were loaded and separated by SDS-PAGE, before being electrophoretically transferred to nitrocellulose filters (semi dry transfer). 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 polyclonal anti-atrial natriuretic peptide (ANP), goat monoclonal anti-GRP78, rabbit polyclonal anti-PERK, rabbit polyclonal anti-Ire-1 α , rabbit polyclonal anti-tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2, mouse polyclonal anti-CHOP, goat polyclonal anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and mouse monoclonal anti- β -tubulin (Santa Cruz Biotechnology, Inc); mouse monoclonal anti-galectin-3 (Affinity Bioreagents, Golden, CO, USA); rabbit polyclonal anti-caspase-12 (BioVision, Inc., Mountain View, CA, USA); and rabbit polyclonal anti-cleaved caspase-3, rabbit polyclonal anti-phospho-p38 MAPK, rabbit polyclonal anti-p38 MAPK, rabbit polyclonal anti-ribosomal S6 kinase (RSK)-1/2/3, rabbit polyclonal anti-p90RSK, rabbit polyclonal anti-MAPK-activated protein kinase (MAPKAPK)-2, rabbit polyclonal anti-phospho-MAPKAPK-2 (Cell Signaling Technology Inc., MA, 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 sham 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

Fig. 2. Pressure overload stimulation resulted in cardiac hypertrophy: Representative photos showing an enlarged heart after the surgery in the WT AB mice and in the DN p38 α AB mice compared with the sham mice. 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 100x magnification to evaluate myocardial fibrosis, stained with TUNEL at 100x magnification to evaluate apoptotic cells and stained with anti-GRP78 at 400x magnification to evaluate GRP78 immunoreactivity.

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.

Results

Myocardial expression of phospho-p38 MAPK and phospho-MAPKAPK-2

As depicted in Fig. 1A-B, cardiac expression of phospho-p38 MAPK was significantly increased in the WT AB mice compared to the WT sham mice; however, cardiac expression of phospho-p38 MAPK was significantly less in the DN p38 α AB mice compared to the WT AB mice. Additionally, p38 MAPK was identified as an upstream kinase of MAPKAPK-2 and p90RSK [18-22]. Immunoblot analysis revealed that phospho-MAPKAPK-2, which is well-known as an *in vivo*

substrate of p38 MAPK, was significantly increased in the WT AB mice compared to the WT sham mice; however, cardiac expression of phospho-MAPKAPK-2 was significantly less in the DN p38 α AB mice compared to the WT AB mice (Fig. 1A and C). Interestingly, there was no significant difference in the expression level of cardiac p90RSK protein among groups (Fig. 1A and D).

Pressure overload elicits cardiac hypertrophy

Seven days after the ascending AB surgery, prominent cardiac enlargement was observed in the WT AB and the DN p38 α AB mice compared with the sham mice (Fig. 2). Although, the aortic-banded DN p38 α mice seemed to develop more cardiac hypertrophy than the aortic-banded WT mice, no significant difference in the HW/BW ratio was observed between the WT AB and DN p38 α AB mice (Fig. 3A).

As depicted in Fig. 2 and 3B, HE staining showed a significant increase in cardiomyocyte diameter in the WT AB mice compared with the WT sham mice ($p < 0.01$) and in the DN p38 α AB mice compared with the DN p38 α sham mice ($p < 0.01$). No significant difference in cardiomyocyte diameter was observed between the aortic-banded WT and aortic-banded DN p38 α mice (Fig.

Fig. 3. (A) Quantification of HW/BW ratio (n=6-8 mice); (B) Quantification of cardiomyocyte diameter (n=3-4 mice); (C) Quantification of myocardial fibrosis (n=3-4 mice); (D) Quantification of TUNEL assay (n=3-4 mice). The black and white bars, respectively, represent the WT and DN p38 α mice. Each bar represents the mean $\pm$ S.E. **p<0.01 vs. WT or DN p38 α sham, #p<0.01 vs. WT AB.

Fig. 4. (A) Western immunoblots of cardiac ANP (n=4 mice) and galectin-3 (n=4 mice) proteins. All bands were normalized against GAPDH; (B) Densitometric analysis of the cardiac hypertrophy marker, ANP expression; (C) Densitometric analysis of the cardiac hypertrophy marker, galectin-3 expression. The black and white bars, respectively, represent the WT and DN p38 α mice. Each bar represents the mean $\pm$ S.E. *p<0.05 vs. WT or DN p38 α sham.

3B). Using Western blot analysis, we also found an increase in cardiac ANP and galectin-3 protein expression levels in the WT AB and DN p38 α AB mice compared with those in the respective sham mice. No significant difference in cardiac galectin-3 or ANP protein expression was observed between the aortic-banded WT and DN p38 α mice (Fig. 4A-C). In addition, myocardial fibrosis was significantly increased in the WT AB mice compared with the WT sham mice (p<0.01). Interestingly, significantly less myocardial fibrosis was observed between the DN p38 α AB and WT AB mice (Fig. 2 and 3C). Collectively, the same degree of hypertrophic stimulation resulted in the same extent of cardiac hypertrophy but induced fibrotic processes to different degrees in the WT and DN p38 α mice.

Apoptotic cells analysis

Seven days after the AB surgery, apoptotic cardiac cells were found in significantly greater numbers in the WT AB mice than in the WT sham mice (Fig. 2 and 3D).

Fig. 5. Cardiac ERS markers during pressure overload stimulus: (A) Western immunoblots of cardiac GRP78 (n=4 mice), PERK (n=4 mice), Ire-1 α (n=4 mice), and TRAF2 (n=4 mice) proteins. All bands were normalized against GAPDH; (B) Densitometric analysis of cardiac GRP78 expression; (C) Densitometric analysis of cardiac PERK expression; (D) Densitometric analysis of cardiac Ire-1 α expression; (E) Densitometric analysis of cardiac TRAF2 expression. In B-E, the black and white bars, respectively, represent the WT and DN p38 α mice. Each bar represents the mean $\pm$ S.E. * p <0.05 and ** p <0.01 vs. WT or DN p38 α sham.

Conversely, significantly less increase in the number of apoptotic cardiac cells was found in the DN p38 α AB mice compared with the WT AB mice (Fig. 2 and 3D).

Expression of cardiac ERS marker

Pressure overload is known to induce cardiac ERS [23]. GRP78 is widely used as a marker of ERS. As depicted in Fig. 2, immunohistochemical analysis demonstrated enhanced immunoreactivity for cardiac GRP78 in the WT AB and DN p38 α AB mice compared with those in their sham counterparts. Using Western blot analysis, we further found that seven days pressure overload significantly induced the cardiac expression of GRP78 protein in the WT AB and DN p38 α AB mice compared with their sham mice (Fig. 5A and B). During the accumulation of misfolded proteins, GRP78 is released to aggregate with transmembrane signaling proteins, including PERK, Ire-1 and activating transcription factor (ATF)-6 and initiates the UPR [1-3]. We further analyzed the expression levels of the ER transmembrane protein PERK and Ire-1 α . Interestingly, Ire-1 α was the only ER transmembrane proteins whose expression was significantly upregulated by the hypertrophic stimulus in the WT and DN p38 α mice compared with their sham

mice (Fig. 5A,C,D). Additionally, the cardiac expression of TRAF2 protein - an adaptor molecule that transduces the ERS signal from Ire-1 α to the downstream pathway - was significantly increased in the WT AB and DN p38 α AB mice compared with their sham mice (Fig. 5A and E). These results suggest that seven days AB surgery activated the cardiac ERS response, at least in part, through the Ire-1 α /TRAF2, but not the PERK pathway, in the WT and DN p38 α mice.

Expression of ERS-associated apoptosis markers

Apoptotic cells were subsequently confirmed by western blot analysis of cleaved caspase-3. As depicted in Fig. 6A and B, the expression level of cardiac cleaved caspase-3 protein, as detected by the presence of the 17 and 19 kDa fragments, was significantly increased in the WT AB mice compared with the WT sham mice. The expression level of this protein tended to increase in the DN p38 α AB mice compared with the DN p38 α sham mice; however, the increase did not attain statistical significance (Fig. 6A and B). Additionally, no significant activation of caspase-12 expression was observed in the WT AB or DN p38 α AB mice compared with their sham mice (Fig. 6A and C).

Fig. 6. (A) Western immunoblots of cardiac cleaved caspase-3 (n=4 mice), caspase-12 (n=4 mice), and CHOP (n=4 mice). All bands were normalized against β -tubulin; (B) Densitometric analysis of cardiac cleaved caspase-3 expression; (C) Densitometric analysis of cardiac caspase-12 expression; (D) Densitometric analysis of cardiac CHOP expression. In B-D, the black and white bars, respectively, represent the WT and DN p38 α mice. Each bar represents the mean $\pm$ S.E. ** p <0.01 vs. WT or DN p38 α sham, # p <0.05 vs. WT AB.

High levels of ERS may activate the ERS-associated apoptosis marker, CHOP, through the formation of Ire-1 α /TRAF2/Apoptosis signal-regulating kinase (ASK)1 complex [11]. A significant increase in the cardiac CHOP protein expression level was observed in the WT AB mice compared with WT sham mice at seven days after the AB surgery (Fig. 6A and D). Conversely, no significant increase in the cardiac CHOP protein expression level was observed in the DN p38 α AB mice compared with the DN p38 α sham mice (Fig. 6A and D). These results suggest that although the hypertrophic stimulus activated the cardiac ERS and UPR responses in WT AB and DN p38 α AB mice, no further apoptotic processes were activated in the DN p38 α AB mice.

Discussion

Our studies have shown that (1) seven days pressure overloading produced by ascending AB surgery significantly induced cardiac hypertrophy and activated the cardiac ERS response through the regulation of the GRP78 and Ire-1 α /TRAF2 pathways in WT and DN p38 α mice and (2) further apoptotic processes as well as CHOP activation were significantly induced in the aortic-banded WT but not the aortic-banded DN p38 α mice.

In this study, we found that aortic-banded DN p38 α MAPK mice had significantly less p38 MAPK and MAPKAPK-2 activity compared to the aortic-banded

WT mice. Interestingly, no significant activation of cardiac p90RSK was observed among groups. These results were consistent with the previous study that MAPKAPK-2 was already shown to be an *in vivo* substrate of p38 MAPK [21] meanwhile activation of p90RSK was mostly mediated by extracellular signal-regulated kinase (ERK)s and JNKs and not by p38 kinase [22]. In addition, we also have reported that MAPKAPK-2 was significantly increased in the doxorubicin-induced WT and DN p38 α MAPK mice. However, doxorubicin-induced DN p38 α MAPK mice had significantly less MAPKAPK-2 activation compared to the doxorubicin-induced WT mice [24].

The ER is a sub-cellular organelle that is involved in the synthesis and folding of secretory/membrane proteins and lipids. Various conditions that perturb cellular energy levels, redox state, and Ca²⁺ concentration can induce ER dysfunction which resulting in the accumulation of unfolded protein, a condition defined as ERS [1-3]. To maintain homeostasis against any ER dysfunction, the ER responds through a complex and coordinated adaptive signaling mechanism called the UPR. However, if the stress is prolonged, signaling switches from pro-survival to pro-apoptotic [2].

In this study, we performed ascending AB surgery since this technique provides a more direct and rapid source of pressure overload on the LV together with a significant degree of hypertrophy after 48 hours [15, 16]. We have shown that seven days pressure overload

induced by AB surgery resulted in similar increases in the HW/BW ratio, cardiomyocyte diameter, and the expression levels of cardiac ANP and galectin-3 in the WT and DN p38 α mice. Interestingly, aortic-banded DN p38 α mice exhibited a less myocardial fibrosis and apoptosis compared with the aortic-banded WT mice. The role of the p38 kinase pathway in cardiac hypertrophy is controversial. In cultured cardiomyocytes, p38 activation induces myocyte hypertrophy and apoptosis and promotes fetal gene expression and cytokine production [8, 25, 26]. However, we have reported that mice expressing a DN cardiac specific p38 α displayed normal hypertrophy but were resistant to fibrosis [14].

Pressure overload stimulation has been also reported to elicit ERS in myocardial cells [23, 27, 28]. We have shown that seven days pressure overload produced similar cardiac ERS responses, as recognized by the expression of GRP78 protein, in the aortic-banded WT and DN p38 α mice compared with the sham mice. Additionally, Ire-1 α /TRAF2 expression was also significantly increased in the aortic-banded WT and DN p38 α mice compared with the sham mice. These results suggest that the partial inhibition of p38 α protein achieved in our model did not attenuate the myocardial ERS response or activation of the Ire-1 α /TRAF2 pathway. These results can be explained through a mechanism by which Ire-1 α signaling is switched to pro-apoptotic signaling. Ire-1 α initiates pro-survival signaling as part of the UPR during ER dysfunction [2], but Ire-1 α is also hypothesized to be able to induce apoptosis [29] and is clearly connected with cell death [30] since active Ire-1 α has been shown to form a complex with the TRAF2 [31, 32]. The UPR in the ER causes GRP78 to induce the release of Ire-1 α , PERK and ATF6. On release, Ire-1 α and PERK oligomerize in the ER membranes. Oligomerized Ire-1 α binds to TRAF2, ASK1 and downstream kinases that activate p38 MAPK and c-Jun N terminal kinase (JNK) [30, 33]. Taken together, our results showed that in the context of the UPR signaling, p38 protein acts as the downstream target of the Ire-1 α /TRAF2 pathway. Therefore, it is understandable that a hypertrophic stimulus that causes ER dysfunction activates the cardiac ERS response to the same extent in WT and DN p38 α mice. However, the partial inhibition of p38 α protein during pressure overload resulted in the attenuation of myocardial apoptosis, caspase-3 cleavage, and CHOP activation in the DN p38 α mice compared with the WT mice. These results support the results of a previous study that found that the post-transcriptional level of CHOP was also enhanced by p38 MAPK [29]. Therefore, it seems that

the partial inhibition of p38 α MAPK during pressure overload protects myocardial cells from further apoptotic processes by attenuating signaling from Ire-1 α /TRAF2 to the downstream protein, CHOP. Additionally, caspase-3 has been reported to be activated in the various duration of pressure overload induction [23, 34–36]. Interestingly, in the model of myocardial injury, selective inhibition of p38 MAPK with a novel small-molecule inhibitor, SD-282, reduced myocardial apoptosis through the reduction of caspase-3 [37]. Therefore, it is speculated that p38 MAPK acts as an upstream of caspase-3 in the regulation of myocardial apoptosis during myocardial injury. However, this result should be further investigated since in the pressure-overloaded CHOP-deficient mice, down-regulation of CHOP resulted in the lower cleaved caspase-3 and smaller number of TUNEL positive cells compared to the pressure-overloaded WT mice [36]. In brief, insignificant difference of cleaved caspase-3 observed in our aortic-banded DN p38 α mice might have been resulted from the direct partial inhibition of p38 MAPK which subsequently inhibited activation of caspase-3 or indirectly from the inhibition of CHOP.

In this study, we did not examine whether p38 MAPK inhibitors are able to attenuate the ERS-associated apoptotic processes through CHOP inhibition during pressure overload. However, accumulating evidence has shown in various conditions that p38 MAPK inhibitors attenuate the phosphorylation of CHOP. The p38 MAPK highly specific inhibitor SB203580 attenuates the stress-inducible phosphorylation of CHOP at Ser⁷⁸ and Ser⁸¹ [11]. Japanese encephalitis virus (JEV) infection has been reported to activate p38 MAPK, which in turn activates the CHOP pathway by triggering the translocation of phosphorylated CHOP into the nucleus to activate gene expression. The blockade of p38 MAPK activity by its specific inhibitor, SB, attenuates the extent of CHOP induction and the subsequent cell death caused by JEV infection [38]. Additionally, in the CD70-induced apoptosis of Epstein-Barr Virus (EBV)-transformed cells, pre-treatment with specific MAPK inhibitors including SP600125 and SB203580 effectively blocked the upregulation of CHOP, growth arrest, and the expression of the DNA damage inducible genes (GADD)-34, and ATF-4 at the mRNA and protein levels [39].

In conclusion, pressure overload stimulation resulted in the significant cardiac hypertrophy accompanied by increased myocardial fibrosis, myocardial apoptosis and activation of the ERS response in WT mice. Additionally, although the same degree of pressure overload stimulation resulted in significant cardiac

hypertrophy and activation of the ERS response in the DN p38 α mice, it produced less myocardial apoptosis and CHOP activation. These results suggest that the partial inhibition of p38 α protein blocks the activation of CHOP-mediated apoptotic processes during pressure overload by partially inhibiting signaling from the Ire-1 α /TRAF2 to its down-stream molecule CHOP. Further studies involving p38 MAPK inhibitors are necessary to further elucidate the mechanisms behind the effects observed in this study.

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References

- Kim I, Xu W, Reed JC: Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* 2008;7:1013-1030.
- Szegezdi E, Logue SE, Gorman AM, Samali A: Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 2006;7:880-885.
- Xu C, Bailly-Maitre B, Reed JC: Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005;115:2656-2664.
- Ushio-Fukai M, Alexander RW, Akers M, Griending KK: p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J Biol Chem* 1998;273:15022-15029.
- Force T, Bonventre JV: Growth factors and mitogen-activated protein kinases. *Hypertension* 1998;31:152-161.
- Lemke LE, Bloem LJ, Fouts R, Esterman M, Sandusky G, Vlahos CJ: Decreased p38 MAPK activity in end-stage failing human myocardium: p38 MAPK α is the predominant isoform expressed in human heart. *J Mol Cell Cardiol* 2001;33:1527-1540.
- Roux PP, Blenis J: ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004;68:320-344.
- Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J, Chien KR: Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem* 1998;273:2161-2168.
- Chen CL, Lin CF, Chang WT, Huang WC, Teng CF, Lin YS: Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway. *Blood* 2008;111:4365-4374.
- Kim DS, Kim JH, Lee GH, Kim HT, Lim JM, Chae SW, Chae HJ, Kim HR: p38 Mitogen-activated protein kinase is involved in endoplasmic reticulum stress-induced cell death and autophagy in human gingival fibroblasts. *Biol Pharm Bull* 2010;33:545-549.
- Wang XZ, Ron D: Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science* 1996;272:1347-1349.
- Liang SH, Zhang W, McGrath BC, Zhang P, Cavener DR: PERK (eIF2 α kinase) is required to activate the stress-activated MAPKs and induce the expression of immediate-early genes upon disruption of ER calcium homeostasis. *Biochem J* 2006;393:201-209.
- Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D: Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell* 2000;5:897-904.
- Zhang S, Weinheimer C, Courtois M, Kovacs A, Zhang CE, Cheng AM, Wang Y, Muslin AJ: The role of the Grb2-p38 MAPK signaling pathway in cardiac hypertrophy and fibrosis. *J Clin Invest* 2003;111:833-841.
- Tarnavski O, McMullen JR, Schinke M, Nie Q, Kong S, Izumo S: Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. *Physiol Genomics* 2004;16:349-360.
- Sari FR, Watanabe K, Widiantoro B, Thandavarayan RA, Harima M, Zhang S, Muslin AJ, Kodama M, Aizawa Y: Partial inactivation of cardiac 14-3-3 protein in vivo elicits endoplasmic reticulum stress (ERS) and activates ERS-initiated apoptosis in ERS-induced mice. *Cell Physiol Biochem* 2010;26:167-178.
- Gurusamy N, Watanabe K, Ma M, Zhang S, Muslin AJ, Kodama M, Aizawa Y: Dominant negative 14-3-3 promotes cardiomyocyte apoptosis in early stage of type I diabetes mellitus through activation of JNK. *Biochem Biophys Res Commun* 2004;320:773-780.
- New L, Zhao M, Li Y, Bassett WW, Feng Y, Ludwig S, Padova FD, Gram H, Han J: Cloning and characterization of RLPK, a novel RSK-related protein kinase. *J Biol Chem* 1999;274:1026-1032.
- Pierrat B, Correia JS, Mary JL, Tomás-Zuber M, Lesslauer W: RSK-B, a novel ribosomal S6 kinase family member, is a CREB kinase under dominant control of p38 α mitogen-activated protein kinase (p38 α MAPK). *J Biol Chem* 1998;273:29661-29671.
- Deak M, Clifton AD, Lucocq LM, Alessi DR: Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J* 1998;17:4426-4441.
- Cuenda A, Cohen P: Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J Biol Chem* 1999;274:4341-4346.
- Zhang Y, Zhong S, Dong Z, Chen N, Bode AM, Ma W, Dong Z: UVA induces Ser381 phosphorylation of p90RSK/MAPKAP-K1 via ERK and JNK pathways. *J Biol Chem* 2001;276:14572-14580.

- 23 Okada K, Minamino T, Tsukamoto Y, Liao Y, Tsukamoto O, Takashima S, Hirata A, Fujita M, Nagamachi Y, Nakatani T, Yutani C, Ozawa K, Ogawa S, Tomoike H, Hori M, Kitakaze M: Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic constriction: possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis. *Circulation* 2004;110:705-712.
- 24 Thandavarayan RA, Watanabe K, Sari FR, Ma M, Lakshmanan AP, Giridharan VV, Gurusamy N, Nishida H, Konishi T, Zhang S, Muslin AJ, Kodama M, Aizawa Y: Modulation of doxorubicin-induced cardiac dysfunction in dominant-negative p38 α mitogen-activated protein kinase mice. *Free Radic Biol Med* 2010;49:1422-1431.
- 25 Zechner D, Thuerauf DJ, Hanford DS, McDonough PM, Glembotski CC: A role for the p38 mitogen-activated protein kinase pathway in myocardial cell growth, sarcomeric organization, and cardiac-specific gene expression. *J Cell Biol* 1997;139:115-127.
- 26 Li M, Georgakopoulos D, Lu G, Hester L, Kass DA, Hasday J, Wang Y: p38 MAP kinase mediates inflammatory cytokine induction in cardiomyocytes and extracellular matrix remodeling in heart. *Circulation* 2005;111:2494-2502.
- 27 Sari FR, Watanabe K, Widyantoro B, Thandavarayan RA, Harima M, Kodama M, Aizawa Y: Sex differences play a role in cardiac endoplasmic reticulum stress (ERS) and ERS-initiated apoptosis induced by pressure overload and thapsigargin. *Cardiovasc Pathol* 2010; in press.
- 28 Zhang ZY, Liu XH, Ye YJ, Sun S, Rong F, Guo XS, Hu WC: C/EBP homologous protein-mediated endoplasmic reticulum stress-related apoptosis pathway is involved in abdominal aortic constriction-induced myocardium hypertrophy in rats. *Sheng Li Xue Bao* 2009;61:161-168.
- 29 Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, Ron D: Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *EMBO J* 1998;17:5708-5717.
- 30 Kim I, Shu CW, Xu W, Shiau CW, Grant D, Vasile S, Cosford ND, Reed JC: Chemical biology investigation of cell death pathways activated by endoplasmic reticulum stress reveals cytoprotective modulators of ASK1. *J Biol Chem* 2009;284:1593-1603.
- 31 Urano F, Bertolotti A, Ron D: IRE1 and efferent signaling from the endoplasmic reticulum. *J Cell Sci* 2000;113:3697-3702.
- 32 Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D: Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 2000;287:664-666.
- 33 Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H: ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 2002;16:1345-1355.
- 34 Moorjani N, Ahmad M, Catarino P, Brittin R, Trabzuni D, Al-Mohanna F, Narula N, Narula J, Westaby S: Activation of apoptotic caspase cascade during the transition to pressure overload-induced heart failure. *J Am Coll Cardiol* 2006;48:1451-1458.
- 35 Philipp S, Pagel I, Höhnelt K, Lutz J, Buttgerit J, Langenickel T, Hamet P, Dietz R, Willenbrock R: Regulation of caspase 3 and Fas in pressure overload-induced left ventricular dysfunction. *Eur J Heart Fail* 2004;6:845-851.
- 36 Fu HY, Okada K, Liao Y, Tsukamoto O, Isomura T, Asai M, Sawada T, Okuda K, Asano Y, Sanada S, Asanuma H, Asakura M, Takashima S, Komuro I, Kitakaze M, Minamino T: Ablation of C/EBP homologous protein attenuates endoplasmic reticulum-mediated apoptosis and cardiac dysfunction induced by pressure overload. *Circulation* 2010;122:361-369.
- 37 Li Z, Ma JY, Kerr I, Chakravarty S, Dugar S, Schreiner G, Protter AA: Selective inhibition of p38 α MAPK improves cardiac function and reduces myocardial apoptosis in rat model of myocardial injury. *Am J Physiol Heart Circ Physiol* 2006;291:H1972-1977.
- 38 Su HL, Liao CL, Lin YL: Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. *J Virol* 2002;76:4162-4171.
- 39 Park GB, Kim YS, Lee HK, Song H, Cho DH, Lee WJ, Hur DY: Endoplasmic reticulum stress-mediated apoptosis of EBV-transformed B cells by cross-linking of CD70 is dependent upon generation of reactive oxygen species and activation of p38 MAPK and JNK pathway. *J Immunol* 2010;185:7274-7284.