Attenuation of CHOP-mediated Myocardial Apoptosis in Pressure-overloaded Dominant Negative p38α Mitogen-activated Protein Kinase Mice

Flori R. Sari  
Niigata University

Bambang Widyantoro  
Kobe University Graduate School of Medicine

Rajarajan A. Thandavarayan  
Niigata University

Meilei Harima  
Niigata University

Arun Prasath Lakshmanan  
Niigata University

See next page for additional authors

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Recommended Citation
Sari, Flori R.; Widyantoro, Bambang; Thandavarayan, Rajarajan A.; Harima, Meilei; Lakshmanan, Arun Prasath; Zhang, Shaosong; Muslin, Anthony J.; Suzuki, Kenji; Kodama, Makoto; and Watanabe, Kenichi, "Attenuation of CHOP-mediated Myocardial Apoptosis in Pressure-overloaded Dominant Negative p38α Mitogen-activated Protein Kinase Mice." Cellular Physiology and Biochemistry.27,5. 487-496. (2011).
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Authors
Flori R. Sari, Bambang Widyantoro, Rajarajan A. Thandavarayan, Meilei Harima, Arun Prasath Lakshmanan, Shaosong Zhang, Anthony J. Muslin, Kenji Suzuki, Makoto Kodama, and Kenichi Watanabe
Attenuation of CHOP-mediated Myocardial Apoptosis in Pressure-overloaded Dominant Negative p38α Mitogen-activated Protein Kinase Mice

Flori R. Sari1,2, Bambang Widyantoro3, Rajarajan A. Thandavarayan1, Meilei Harima1, Arun Prasath Lakshmanan1, Shaosong Zhang4, Anthony J. Muslin5, Kenji Suzuki6, Makoto Kodama7 and Kenichi Watanabe1

1Department of Clinical Pharmacology, Niigata University of Pharmacy and Applied Life Sciences, 265-1 Higashijima, Niigata City, 2Department of Pharmacology, Faculty of Medicine and Health Sciences, Syarif Hidayatullah Jakarta, State Islamic University, South Jakarta, 3Division of Cardiovascular Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki, Chuo, Kobe, 4Lightlab Imaging, Inc., One Technology Park Drive, Westford, 5Center for Cardiovascular Research, John Milliken Department of Internal Medicine, Washington University School of Medicine, St. Louis, 6Department of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Science, 1-754 Asahimachi, Niigata, 7First Department of Internal Medicine, Niigata University Graduate School of Medical and Dental Science, 1-754 Asahimachi, Niigata

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 p38α MAPK in ERS-associated apoptosis by subjecting transgenic mice displaying cardiac specific dominant negative (DN) mutant p38α 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 p38α 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 p38α mice. Conclusion: Partial inhibition of p38α protein and its downstream molecule, tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2 in the WT and DN p38α 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 p38α mice. Conclusion: Partial inhibition of p38α protein blocked the activation of CHOP-mediated apoptotic processes during pressure overload by partially inhibiting signaling from the IRE-1α/TRAF2 to its downstream molecule, CHOP.

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

1α and its downstream molecule, tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2 in the WT and DN p38α mice compared with the sham mice.
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 p38α MAPK in ERS-associated apoptosis, we subjected transgenic mice that displayed cardiac specific dominant negative (DN) mutant p38α MAPK over-expression to seven days pressure overload.

Materials and Methods

Generation of DN p38α MAPK mice

Transgenic DN p38α 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 p38α 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 p38α MAPK mice (DN p38α 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 p38α 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 Finetech 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 Finetech Co) were cut into 4 μm 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).

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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 μg 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, rabbit polyclonal anti-tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2, rabbit polyclonal anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and mouse monoclonal anti-CHOP ; goat polyclonal anti-ribosomal S6 kinase (RSK)-1/2/3, rabbit polyclonal anti-MAPK-activated protein kinase (MAPKAPK)-2, rabbit polyclonal anti-caspase-12, 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

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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 p38α 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 p38α 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 p38α AB mice compared with the sham mice (Fig. 2). Although, the aortic-banded DN p38α 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 p38α 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 p38α AB mice compared with the DN p38α sham mice (p<0.01). No significant difference in cardiomyocyte diameter was observed between the aortic-banded WT and aortic-banded DN p38α mice (Fig. 3B).

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Using Western blot analysis, we also found an increase in cardiac ANP and galectin-3 protein expression levels in the WT AB and DN p38α AB mice compared with those in the respective sham mice. No significant difference in cardiac galectin-3 or ANP expression was observed between the aortic-banded WT and DN p38α 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 p38α 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 p38α 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).
Conversely, significantly less increase in the number of apoptotic cardiac cells was found in the DN p38α 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 p38α 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 p38α 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-1α. Interestingly, Ire-1α was the only ER transmembrane proteins whose expression was significantly upregulated by the hypertrophic stimulus in the WT and DN p38α 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-1α to the downstream pathway - was significantly increased in the WT AB and DN p38α 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-1α/TRAF2, but not the PERK pathway, in the WT and DN p38α 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 p38α AB mice compared with the DN p38α 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 p38α AB mice compared with their sham mice (Fig. 6A and C).
High levels of ERS may activate the ERS-associated apoptosis marker, CHOP, through the formation of Ire-1α/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 p38α AB mice compared with the DN p38α 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 p38α AB mice, no further apoptotic processes were activated in the DN p38α 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-1α/TRAF2 pathways in WT and DN p38α 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 p38α mice.

In this study, we found that aortic-banded DN p38α 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 p38α MAPK mice. However, doxorubicin-induced DN p38α 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 p38α mice. Interestingly, aortic-banded DN p38α 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 p38α 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 p38α mice compared with the sham mice. Additionally, Ire-1α/TRA2F expression was also significantly increased in the aortic-banded WT and DN p38α mice compared with the sham mice. These results suggest that the partial inhibition of p38α protein achieved in our model did not attenuate the myocardial ERS response or activation of the Ire-1α/TRA2F pathway. These results can be explained through a mechanism by which Ire-1α signaling is switched to pro-apoptotic signaling. Ire-1α initiates pro-survival signaling as part of the UPR during ER dysfunction [2], but Ire-1α is also hypothesized to be able to induce apoptosis [29] and is clearly connected with cell death [30] since active Ire-1α 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-1α, PERK and ATF6. On release, Ire-1α and PERK oligomerize in the ER membranes. Oligomerized Ire-1α 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-1α/TRA2F 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 p38α mice. However, the partial inhibition of p38α protein during pressure overload resulted in the attenuation of myocardial apoptosis, caspase-3 cleavage, and CHOP activation in the DN p38α 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 p38α MAPK during pressure overload protects myocardial cells from further apoptotic processes by attenuating signaling from Ire-1α/TRA2F 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, downregulation 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 p38α 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 p38α mice, it produced less myocardial apoptosis and CHOP activation. These results suggest that the partial inhibition of p38α protein blocks the activation of CHOP-mediated apoptotic processes during pressure overload by partially inhibiting signaling from the Ire-1/ 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.

Acknowledgements

We thank Sayaka Egawa, Wawaimuli Arozal, Vijayakumar Sukumaran, Yoshiyasu Kobayashi, Vivian Soetikno, Somasundaram Arumugam for their assistance with this research.

This research was supported by the grants from the Yujin Memorial Grant; the Ministry of Education, Science, Sports, and Culture of Japan; and the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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Cell Physiol Biochem 2011;27:487-496


