Heart Failure

Ablation of C/EBP Homologous Protein Attenuates Endoplasmic Reticulum–Mediated Apoptosis and Cardiac Dysfunction Induced by Pressure Overload

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Background—Apoptosis may contribute to the development of heart failure, but the role of apoptotic signaling initiated by the endoplasmic reticulum in this condition has not been well clarified.

Methods and Results—In myocardial samples from patients with heart failure, quantitative real-time polymerase chain reaction revealed an increase in messenger RNA for C/EBP homologous protein (CHOP), a transcriptional factor that mediates endoplasmic reticulum–initiated apoptotic cell death. We performed transverse aortic constriction or sham operation on wild-type (WT) and CHOP-deficient mice. The CHOP-deficient mice showed less cardiac hypertrophy, fibrosis, and cardiac dysfunction compared with WT mice at 4 weeks after transverse aortic constriction, although the contractility of isolated cardiomyocytes from CHOP-deficient mice was not significantly different from that in the WT mice. In the hearts of CHOP-deficient mice, phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which may reduce protein translation, was enhanced compared with WT mice. In the hearts of WT mice, CHOP-increased apoptotic cell death with activation of caspase-3 was observed at 4 weeks after transverse aortic constriction. In contrast, CHOP-deficient mice had less apoptotic cell death and lower caspase-3 activation at 4 weeks after transverse aortic constriction. Furthermore, the Bcl2/Bax ratio was decreased in WT mice, whereas this change was significantly blunted in CHOP-deficient mice. Real-time polymerase chain reaction microarray analysis revealed that CHOP could regulate several Bcl2 family members in failing hearts.

Conclusions—We propose the novel concept that CHOP, which may modify protein translation and mediate endoplasmic reticulum–initiated apoptotic cell death, contributes to development of cardiac hypertrophy and failure induced by pressure overload. (Circulation. 2010;122:361-369.)

Key Words: apoptosis ■ endoplasmic reticulum ■ heart failure ■ hypertrophy

The endoplasmic reticulum (ER) is classically characterized as an organelle that participates in the folding of membrane proteins and secretory proteins. Various cellular stresses, including ischemia, hypoxia, heat shock, genetic mutation, oxidative stress, and increased protein synthesis, can lead to impairment of ER function. Stimuli that cause ER dysfunction are collectively known as ER stress. When ER stress occurs, various ER stress sensor proteins activate a transcriptional and translational response that is known as the unfolded protein response to cope with the accumulation of unfolded or misfolded proteins in the ER lumen. After the onset of ER stress, eukaryotic translation initiation factor 2α (eIF2α) is phosphorylated to repress global protein synthesis. When ER stress is excessive and/or prolonged, however, apoptotic signals are initiated by the ER, including induction of C/EBP homologous protein (CHOP), activation of Jun N-terminal kinase (JNK), and cleavage of caspase-12. Previous studies have demonstrated the morphological development of the ER in hypertrophic and failing human hearts. Because the morphological development of the ER indicates the overload to the ER, the unfolded protein response should occur in diseased hearts. However, the...
molecular signaling mechanisms involved in the unfolded protein response have not been fully identified in diseased hearts. Recently, we have demonstrated that expression of ER chaperones was increased and CHOP was induced in experimental heart failure.9 CHOP has been identified as an ER-initiated proapoptotic signal that plays an important role in the pathophysiology of diabetes mellitus and neurodegenerative diseases.3,10,11 Furthermore, CHOP can also directly regulate death effectors such as Bcl2, which is one of key determinants of cell death or survival.12 Although the apoptosis of cardiomyocytes may contribute to the development of heart failure,13–16 the role of ER-initiated apoptosis in the pathophysiology of heart failure remains unclear. In the present study, we investigated ER stress signaling in human hearts. We also performed in vivo studies to clarify the pathophysiological role of CHOP in the development of cardiac hypertrophy and failure and examined the potential downstream signaling of CHOP in pressure-overloaded hearts of mice.

Methods

Materials
Antibodies for CHOP, Bax, Bcl2, ATF4, ATP5a, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif); antibodies for phospho-SAPK/JNK, SAPK/JNK, phospho-eIF2α, eIF2α, and cleaved caspase-3 were obtained from Cell Signaling Technology, Inc (Danvers, Mass). Isoproterenol and the antibody for caspase-12 were obtained from Sigma Chemical Corp (St. Louis, Mo). Antibodies for BiP and GAPDH were purchased from Assay Designs, Inc (Ann Arbor, Mich) and Millipore Corp (Billerica, Mass), respectively.

Human Heart Samples
Human heart samples were studied according to the protocol approved by the Institutional Review boards of the National Cardiovascular Center (No. 14 to 18) and Hayama Heart Center. For quantitative real-time reverse-transcription polymerase chain reaction (PCR), we used surgical samples of myocardium removed from 12 patients with dilated cardiomyopathy and 3 patients with ischemic cardiomyopathy who underwent left ventriculoplasty at the Hayama Heart Center. Six control heart samples for quantitative real-time PCR were obtained from Sigma Chemical Corp (St. Louis, Mo). Antibodies for BiP and GAPDH were purchased from爱好 Designs, Inc (Ann Arbor, Mich) and Millipore Corp (Billerica, Mass), respectively.

Animal Preparation
Mice lacking the CHOP gene were generated on a C57BL/6 background as described previously.17 Experiments were performed with CHOP-deficient mice and littermate control mice. All procedures were done in accordance with the guiding principles of Osaka University School of Medicine with regard to animal care and the “Position of the American Heart Association on Research Animal Use.”

Transverse Aortic Constriction
Pressure overload of the heart was induced in 10-week-old male mice (20 to 25 g) by transverse aortic constriction (TAC) as described previously.18,19

Immunohistological Analysis
Immunohistological analysis was performed as described previously.18,19

Echocardiography
Cardiac function was assessed by Doppler echocardiography with a 15-MHz transducer (Philips, SONOS5500, Eindhoven, the Netherlands).18,19

Preparation of Neonatal Rat Cardiomyocytes
Primary cardiomyocyte cultures were prepared from neonatal rat hearts.20 We used short interfering RNA (siRNA) (cocktail containing equal amounts of the 3 types of siRNA—5′-CGAAGAGGAGAAGAC-AAA-3′, 5′-GGAAACACGGAGCUGAGGA-3′, and 5′-GGGACGAGGGUAGACCAA-3′) to knock down CHOP messenger RNA (mRNA) as described previously.20 Cardiomyocytes were stained with rhodamine-phalloidin (Invitrogen Corp, Carlsbad, Calif).

Measurement of Contractility With Isolated Adult Mice Cardiomyocytes
Adult cardiomyocytes were isolated from 8-week-old wild-type (WT) or CHOP knockout mice and were stored in Hanks buffer (Invitrogen Corp) containing 1.2 mmol/L CaCl2. Field stimulation at 1-Hz pacing rate was done with 5-ms square pulses of constant voltage at 20% above threshold.31 Changes in cell length during contracting and relaxing were recorded with an inverted microscopy (Olympus IX81, Olympus Corp, Tokyo, Japan) and analyzed with Metamorph software (Molecular Devices Corp, Tokyo, Japan). Cell shortening was calculated as follows: (relaxing length — contracting length)/relaxing length.

Quantitative Real-Time PCR
Quantitative real-time PCR of human heart tissue was performed according to the Omniscript Reverse Transcription Handbook (Qiagen Inc, Valencia, Calif). The primers and probes used for quantification of BiP, ATF4, CHOP, and GAPDH were all designed according to the manufacturer’s protocol (Applied Biosystems, Foster City, Calif; https://www.appliedbiosystems.com/catalog/). Quantitative real-time PCR for the detection of GADD34 was performed with QuantiTect SYBR Green Kit (Qiagen, Inc) with the following primers: GADD34: forward, ATCTCTGGAACAGTCTACCAAGCC; reverse, TAGCCAAACCTTCCCAGCCTTATAC; GAPDH: forward, CATCAAGGACCTTCATTGACCTAACA; reverse, TCCACGATGCAAAGTTGTGCATGGATGACC. Quantitative real-time PCR was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) by the relative standard curve method. The reaction was performed at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The amount of each product was determined from the relative standard curves constructed with serial dilutions of the control cDNA.

Real-Time PCR Microarray Analysis
We isolated RNA from the hearts of WT and CHOP-deficient mice at 4 weeks after TAC or sham operation. Then, the synthesis of cDNA and comparison of relative gene expressions for 15 Bcl2 family members were performed with the RT2 First Strand Kit and a mouse PCR array (Superarray Bioscience Corp, Frederick, Md). Samples from 3 sham-operated and 3 pressure-overloaded hearts at 4 weeks after operation were compared in WT and CHOP-deficient mice. The average cycle threshold (Ct) was calculated for each Bcl2 family gene and the housekeeping genes (GAPDH and actin), and the ΔCt (CtBcl2 family gene – Ctaverage of GAPDH and actin) was determined.22 The values were expressed as the 2–ΔΔCt and, data of sham-operated and pressure-overloaded hearts were analyzed with 2–ΔΔCt method.22

Immunoblotting Analysis
Immunoblotting analysis was performed as described previously.19

Apopotic Cell Assay
The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed as described previously.19 The number of TUNEL-positive cells was expressed as a percentage of total cells.
Statistical Analysis
Data are shown as mean±SEM when normally distributed and as median and interquartile range when nonnormally distributed. The Mann-Whitney U test was used to compare the levels of ATF4, BiP, and CHOP mRNA between control and failing hearts. The results of echocardiographic parameters, quantitative analysis of immunoblotting, and quantitative real-time PCR were compared by 2-way ANOVA followed by the Bonferroni procedure as a posthoc test. Real-time PCR microarray data were analyzed by the 2-ΔΔCT method with RT2 profiler PCR array data analysis software (http://www.superarray.com/pcrarraydataanalysis.php). For all analyses, values of P<0.05 were accepted as statistically significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Activation of ER Stress Signaling in Failing Human Hearts
ER stress leads to morphological development of the ER and induces the unfolded protein response that increases the expression of ER chaperones such as BiP.7,8 The BiP mRNA level was significantly higher in the hearts of 15 patients with heart failure (Table 1) compared with the 6 control subjects (Figure 1A). Increased expression of this ER-resident chaperone indicates that ER stress occurs in failing human hearts. ATF4, a transcriptional factor that regulates CHOP expression,1,2 and CHOP mRNA levels were also significantly higher in the failing human hearts compared with control hearts (Figure 1B and 1C).

Table 1. Clinical Characteristics of 15 Patients With Heart Failure

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>BNP, pg/mL</th>
<th>NYHA Class</th>
<th>LVEF, %</th>
<th>LVDd, mm</th>
<th>LVDs, mm</th>
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<td>1</td>
<td>68</td>
<td>M</td>
<td>ICM</td>
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<td>33</td>
<td>100</td>
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<td>2</td>
<td>50</td>
<td>F</td>
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<td>417</td>
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<td>ICM</td>
<td>321</td>
<td>III</td>
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<td>F</td>
<td>DCM</td>
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<td>III</td>
<td>33</td>
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<td>15</td>
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<td>DCM</td>
<td>1736</td>
<td>IV</td>
<td>26</td>
<td>82</td>
<td>74</td>
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BNP indicates brain-type natriuretic peptide; NYHA, New York Heart Association; LVEF, LV ejection fraction; LVDd, LV end-diastolic dimension; LVDs, LV end-systolic dimension; ICM, ischemic cardiomyopathy; and DCM, dilated cardiomyopathy.

Figure 1. Activation of ER stress signaling in failing human hearts. Quantitative analysis of BiP (A), ATF4 (B), and CHOP (C) mRNA by quantitative real-time PCR in control (CTL; n=6) and failing (congestive heart failure [CHF]; n=15) human hearts. Clinical characteristics of the 15 patients with heart failure are shown in Table 1. Results of real-time reverse-transcription PCR were normalized by GAPDH expression. The central line in each box denotes the median value; the lower and upper boundaries of the box denote interquartile range; and the lower and upper error bars denote minimum and maximum values, respectively.

Attenuation of Cardiac Hypertrophy and Dysfunction Induced by Pressure Overload in CHOP-Deficient Mice
Because CHOP is involved in ER-initiated apoptotic signaling and was markedly induced in failing human hearts, we investigated its pathophysiological role in the development of heart failure. WT and CHOP-deficient mice were subjected to pressure overload by TAC. There were no significant differences in baseline body weight (23.2±0.1 versus 23.4±0.1 g) and hemodynamic parameters, including heart rate (649±10 versus 652±12 bpm) and systolic blood pressure (112±4
versus 111±3 mm Hg), between WT mice and CHOP-deficient mice. On gross examination, CHOP-deficient mice showed less enlargement of the heart compared with WT mice at 4 weeks after TAC (Figure 2A). The ratios of heart weight to body and lung weight to body weight at 4 weeks after TAC were both significantly smaller in CHOP-deficient mice than in WT mice (Figure 2B). On microscopic examination, CHOP-deficient mice showed less cardiac hypertrophy (Figure 2C) and cardiac fibrosis (Figure 2D) compared with WT mice at 4 weeks after sham operation and TAC, respectively. E, Representative pictures showing cultured rat neonatal cardiomyocytes stained with rhodamine-phalloidin (left), representative immunoblotting of CHOP (top right), and quantitative analysis of cell size (bottom right). Cardiomyocytes were treated with siRNA for CHOP (siCHOP) or firefly luciferase from Photinus pyralis (siCTL) as a negative control (60 nmol/L) 4 hours after cardiomyocyte isolation. The immunoblotting and histological analyses were performed 24 hours after treatment with isoproterenol (ISO; 10 μmol/L). *P<0.05 vs control (CTL) and isoproterenol treatments, respectively.
Echocardiography revealed that the baseline left ventricular (LV) end-diastolic and systolic dimensions were not significantly different between WT mice and CHOP-deficient mice (Table 2). However, both the LV end-diastolic and end-systolic dimensions were significantly smaller in CHOP-deficient mice than in WT mice at 4 and 8 weeks after TAC (Table 2). Moreover, LV posterior wall thickness and calculated LV mass in CHOP-deficient mice were smaller than in WT mice. These findings suggest that CHOP plays an important role in the development of cardiac hypertrophy and failure by pressure overload in mice.

ER Stress Signaling in Pressure-Overloaded Hearts of WT and CHOP-Deficient Mice

Next, we investigated ER stress signaling in the pressure-overloaded hearts of WT mice and CHOP-deficient mice.

Table 2. Echocardiographic Parameters in WT and CHOP-Deficient Mice at 4 and 8 Weeks After Sham Operation or TAC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>CHOP&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Sham (n=8)</td>
<td>TAC at 4 wk (n=8)</td>
<td>TAC at 8 wk (n=4)</td>
</tr>
<tr>
<td></td>
<td>Sham (n=6)</td>
<td>TAC at 4 wk (n=9)</td>
<td>TAC at 8 wk (n=3)</td>
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<td>LVDd, mm</td>
<td>3.10±0.06</td>
<td>3.50±0.07</td>
<td>4.30±0.52</td>
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<td>3.80±0.05</td>
<td>4.50±0.60</td>
<td>5.00±0.70</td>
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<td>LVDs, mm</td>
<td>0.64±0.02</td>
<td>0.80±0.02</td>
<td>0.94±0.03</td>
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<td>0.64±0.03</td>
<td>0.80±0.02</td>
<td>0.87±0.04</td>
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<td>LVFS, %</td>
<td>60.7±1.1</td>
<td>35.0±1.0</td>
<td>22.3±6.9</td>
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<td>62.2±0.9</td>
<td>42.6±1.1</td>
<td>39.8±0.8</td>
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<tr>
<td>LVEF, %</td>
<td>91.4±0.6</td>
<td>63.4±2.7</td>
<td>45.5±11.6</td>
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<td>91.8±0.5</td>
<td>74.6±1.1</td>
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<td>LV mass, mg</td>
<td>46.3±2.4</td>
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<td>111.8±7.5</td>
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<td>46.6±2.4</td>
<td>71.4±2.5</td>
<td>89.7±10.2</td>
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</table>

LVd indicates LV end-diastolic dimension; LVDs, LV end-systolic dimension; LVFS, LV fractional shortening; and LVEF, LV ejection fraction. Results are presented as mean±SEM.

*P<0.05 versus WT sham operation.
†P<0.05 versus WT TAC at 4 weeks.
‡P<0.05 versus WT TAC at 8 weeks.

Echocardiography revealed that the baseline left ventricular (LV) end-diastolic and systolic dimensions were not significantly different between WT mice and CHOP-deficient mice (Table 2). However, both the LV end-diastolic and end-systolic dimensions were significantly smaller in CHOP-deficient mice than in WT mice at 4 and 8 weeks after TAC (Table 2). Moreover, LV posterior wall thickness and calculated LV mass in CHOP-deficient mice were smaller than in WT mice. These findings suggest that CHOP plays an important role in the development of cardiac hypertrophy and failure by pressure overload in mice.

ER Stress Signaling in Pressure-Overloaded Hearts of WT and CHOP-Deficient Mice

Next, we investigated ER stress signaling in the pressure-overloaded hearts of WT mice and CHOP-deficient mice. We

Figure 3. ER stress signaling in pressure-overloaded hearts of WT and CHOP-deficient mice. A, Representative immunoblotting (p-eIF2/total eIF2, total elf2α, ATF4, and GAPDH) of ER stress signaling in the hearts of WT and CHOP-deficient mice after sham or TAC operation. The intensity of bands for molecules involved in ER-initiated signaling was quantified from 3 independent experiments by densitometry. B, Quantitative real-time PCR analysis of GADD34 in the hearts of WT and CHOP-deficient mice after sham or TAC operation. C, Schematic diagram of CHOP ablation to attenuate cardiac hypertrophy and apoptosis. D, Representative immunoblotting analysis of ER-initiated apoptosis regulatory proteins in the hearts of WT and CHOP-deficient mice after sham or TAC operation. The intensity of bands for Bip and ER-initiated apoptosis regulatory proteins was quantified from 4 independent experiments by densitometry. Quantitative analysis showed the induction of Bip and CHOP but no increase in caspase-12 cleavage or JNK phosphorylation in the hearts of WT mice after TAC. Results are presented as mean±SEM. *P<0.05 vs WT mice at 4 weeks after sham operation and TAC, respectively.
found that phosphorylation of eIF2α and expression of ATF4, either of which is an upstream signal of CHOP, were increased in hearts after TAC compared with those after sham operation in WT mice (Figure 3A). Expression of CHOP and its downstream target GADD34 was also increased by pressure overload in WT mice (Figure 3A and 3B). CHOP directly activates GADD34, which negatively regulates the phosphorylation of eIF2α and thus increases protein synthesis.3,23,24 Consistently, in the hearts of CHOP-deficient mice, the increase in GADD34 caused by pressure overload was blunted, which led to enhanced phosphorylation of eIF2α and increased ATF4 (Figure 3A through 3C).

Among the factors involved in ER-initiated apoptotic signaling, caspase-12 or JNK was not activated by pressure overload in the hearts of WT and CHOP-deficient mice at 4 weeks after TAC (Figure 3D). This result was consistent with our previous findings.9

Bcl2 Family Genes in Sham-Operated and Pressure-Overloaded Hearts of WT and CHOP-Deficient Mice

The total Bax protein level was increased and the Bcl2 protein level was decreased in the pressure-overloaded hearts of WT mice, leading to a reduction in the Bcl2/Bax ratio (Figure 4A and 4B). We also observed an increase in Bax protein in the mitochondrial fraction from pressure-overloaded hearts of WT mice (Figure 4C). Furthermore, caspase-3 was activated and the number of TUNEL-positive cells was increased in pressure-overloaded hearts of WT mice (Figure 4A and 4D). In contrast, the levels of Bcl2, total Bax, and mitochondrial Bax protein in the hearts of CHOP-deficient mice with pressure overload did not differ from those in sham-operated mice (Figure 4A through 4C). The level of cleaved caspase-3 was lower and the number of TUNEL-positive cells was smaller in the hearts of CHOP-deficient mice than in WT mice (Figure 4D). In the isolated adult cardiomyocyte from WT and CHOP-deficient mice, there were no significant differences in cardiac contractility under pacing conditions (Figure 4E and the online-only Data Supplement). These findings indicate that CHOP, a transcription factor involved in ER-initiated apoptotic signaling, has a role in mitochondria-dependent apoptosis in hearts subjected to pressure overload.

Finally, we assessed the role of CHOP in the expression of Bcl2 family genes in sham-operated and pressure-overloaded hearts from both WT and CHOP-deficient mice. In sham-operated hearts, none of the 15 Bcl2 family gene expressions showed differences between WT mice and CHOP-deficient mice (Table 3).
Table 3. Gene Expressions of Bcl2 Family Members in Hearts From WT and CHOP-Deficient Mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT Sham (2^{-ΔΔCt}×10^3)</th>
<th>TAC (2^{-ΔΔCt}×10^3)</th>
<th>TAC/Sham</th>
<th>CHOP^{-/-} Sham (2^{-ΔΔCt}×10^3)</th>
<th>TAC (2^{-ΔΔCt}×10^3)</th>
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<td>Bad</td>
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<td>12.8±1.3</td>
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<td>Bax</td>
<td>20.7±1.8</td>
<td>35.3±2.4</td>
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<td>Bid</td>
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<td>1.0±0.1</td>
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<td>Bag1</td>
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<td>0.8</td>
<td>180.1±15.9</td>
<td>167.0±12.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Bcl2</td>
<td>5.8±0.5</td>
<td>2.6±0.1</td>
<td>0.5*</td>
<td>5.2±0.2</td>
<td>6.9±1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Bcl2l1</td>
<td>15.0±0.4</td>
<td>10.1±0.8</td>
<td>0.7*</td>
<td>20.1±1.3</td>
<td>16.4±0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Bcl210</td>
<td>Undetected</td>
<td>Undetected</td>
<td>…</td>
<td>Undetected</td>
<td>Undetected</td>
<td>…</td>
</tr>
<tr>
<td>Bcl2l2</td>
<td>8.7±1.3</td>
<td>5.1±0.5</td>
<td>0.6*</td>
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<td>4.4±0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Bnip2</td>
<td>60.7±6.2</td>
<td>70.5±5.2</td>
<td>1.2</td>
<td>71.2±1.9</td>
<td>67.8±4.5</td>
<td>1.0</td>
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<tr>
<td>Mcl1</td>
<td>179.6±6.3</td>
<td>276.1±16.2</td>
<td>1.5*</td>
<td>184.9±10.0</td>
<td>237.0±3.3</td>
<td>1.3*</td>
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</table>

Comprehensive analysis of the gene expressions of 15 Bcl2 family members in the sham-operated or pressure-overloaded hearts from WT and CHOP-deficient mice. Values are expressed as 2^{-ΔΔCt}×10^3, and data of sham-operated and pressure-overloaded hearts were analyzed with the 2^{-ΔΔCt} method. Results are presented as mean±SEM.

*P<0.05 versus gene expression at 4 weeks after sham operation on corresponding mice (n=3).

Consistent with the alterations in Bcl2 and Bax protein levels, we confirmed that Bcl2 and Bax gene expressions decreased and increased in the pressure-overloaded hearts of WT mice, respectively (Table 3). In addition, we found significant changes in the expressions of other Bcl2 family member genes in pressure-overloaded hearts of WT mice compared with sham-operated ones: an increase in 3 proapoptotic genes (Bid, Bnip3l, and Bok) and 1 antiapoptotic gene (Mcl1) and a decrease in 2 antiapoptotic genes (Bcl2l1 and Bcl2l2). In CHOP-deficient mice, except for Bid, Bok, and Mcl1, the expressions of Bcl2 family members did not change in pressure-overloaded hearts compared with sham-operated ones. Interestingly, in addition to Bcl2 and Bax, the expressions of Bnip3l, Bcl2l1, and Bcl2l2 in pressure-overloaded hearts differed between WT and CHOP-deficient mice. These findings suggest that ER-initiated apoptotic signaling via CHOP may mediate cardiac myocyte apoptosis by reducing the Bcl2/Bax ratio and/or by altering the expression of other members of the Bcl2 family to trigger mitochondria-initiated apoptosis.

**Discussion**

The present study confirmed an increase in the expression of BiP, an ER-resident chaperone that facilitates protein folding, in failing human hearts.9 When ER stress is excessive and/or prolonged, apoptotic signals are initiated by the ER, including induction of CHOP, activation of JNK, and cleavage of caspase-12.25–27 We confirmed that mRNA levels of ATF4 and CHOP were increased in failing human hearts, suggesting that CHOP could be important for proapoptotic signaling initiated by the ER in human heart failure. Experimental studies showed that CHOP has a critical role in dilated cardiomyopathy caused by aberrant ER quality control and in ischemic neuronal death,28,29 suggesting that CHOP may be an important molecule in human heart and other diseases.

Therefore, we investigated the pathophysiological role of CHOP in the development of heart failure by using CHOP-deficient mice.17 We previously demonstrated that cardiac dysfunction is induced in mice by pressure overload at 4 weeks after TAC.9 Pressure overload caused by TAC increased the ratios of heart weight to body weight and of lung weight to body weight in WT mice, whereas these changes were partially but significantly attenuated in CHOP-deficient mice. In addition, histological analysis revealed that both cardiac hypertrophy and fibrosis in CHOP-deficient mice were partially but significantly attenuated in CHOP-deficient mice.17 We previously demonstrated that cardiac hypertrophy resulting from pressure overload.

CHOP regulates the expression of GADD34, which negatively regulates the phosphorylation of eIF2α. Enhanced phosphorylation of eIF2α reduces protein translation30 and has been reported to mediate the inhibition of protein synthesis in the rat liver by vasopressin and the rat brain by essential amino acid deficiency.31,32 Under CHOP-deficient conditions, decreased expression of GADD34 in pressure-overloaded hearts may lead to enhanced phosphorylation of eIF2α and
decreased protein synthesis. Thus, increased phosphorylation of eIF2α in hearts with pressure overload in CHOP-deficient mice is likely to contribute to the prevention of cardiac hypertrophy through the suppression of protein synthesis. Interestingly, pressure overload to hearts specifically activated CHOP but not caspase-12 or JNK. The promoter region of the CHOP gene contains binding sites for all of the major inducers of the unfolded protein response, including ATF4, ATF6, and XBP-1; these transcriptional factors are also involved in the induction of CHOP.33 It will be important to clarify the selective activation of CHOP in future investigations.

Overexpression of CHOP leads to a decrease in Bcl2 protein, whereas overexpression of Bcl2 blocks CHOP-induced apoptosis.12,34 In addition, overexpression of CHOP leads to translocation of Bax protein from the cytosol to the mitochondria.35 Thus, the CHOP-mediated death signal is finally transmitted to the mitochondria, leading to activation of caspase-3.27,36 In the present study, expression of Bax protein was increased and Bcl2 protein was decreased in the hearts of WT mice after TAC, consistent with previous data.37 In contrast, these changes in apoptosis-regulating proteins did not occur in hearts of CHOP-deficient mice. These findings suggest that prolonged pressure overload leads to changes in apoptosis-regulating proteins via a CHOP-dependent pathway. Consequently, caspase-3 cleavage was reduced and the number of TUNEL-positive cells was smaller in the hearts of CHOP-deficient mice compared with WT mice at 4 weeks after TAC. Because there were no significant differences in contractility of isolated cardiomyocytes in WT and CHOP-deficient mice, it was likely that the improvement in cardiac function after TAC in CHOP-deficient mice was due to less apoptotic cell death.

Moreover, we performed real-time PCR microarray analysis for 15 Bcl2 family members. We found that the expressions of Bcl2 family member genes in sham-operated hearts were not significantly different between WT mice and CHOP-deficient mice. In pressure-overloaded hearts of WT mice, several Bcl2 family genes, including 4 proapoptotic genes and 4 antiapoptotic genes, were altered. Clarification of the role of each Bcl2 family gene in the development of heart failure is needed in our future studies. Interestingly, in addition to Bcl2 and Bax, we found the difference in the expressions of 3 Bcl2 family genes in pressure-overloaded hearts between WT mice and CHOP-deficient mice: Bnip3l, Bcl2l1, and Bcl2l2. Bnip3l, also referred to as Nix, is induced in cardiac hypertrophy and mediates cardiomyocyte apoptosis.38

Consistent with this report, the increase in Bnip3l expression was blunted in pressure-overloaded hearts of CHOP-deficient mice. Furthermore, expressions of both Bcl2l1 and Bcl2l2 were decreased in pressure-overloaded hearts of WT but not CHOP-deficient mice. Bcl2l1 has been reported to be decreased in hypertrophic and failing hearts after pressure overload.39,40 Although the role of Bcl2l2 in cardiomyocytes remains unclear, it is thought to play an important protective role in neurons and in the diseased brain.40 Because the expressions of Bid, Bok, and Mcl1 changed in a similar way between WT mice and CHOP-deficient mice, they may not contribute to the differences in cardiac dysfunction between WT mice and CHOP-deficient mice. These findings suggest that ER stress initiates CHOP-dependent apoptotic signaling, which finally leads to activation of mitochondria-dependent apoptotic signaling via several Bcl2 family members and contributes to heart failure induced by pressure overload. However, we need to carefully consider the difference in gene expression because of the small number of samples. Furthermore, because CHOP mediates apoptosis through the perturbation of the cellular redox state by depletion of intracellular glutathione and through protein-protein interactions,35,41 we also need to consider whether CHOP influences mitochondria-independent apoptosis in failing hearts.

### Conclusion

The present findings suggest that CHOP may be a logical target for the development of drugs to prevent cardiac hypertrophy and cardiomyocyte apoptosis in failing hearts.

### Acknowledgment

We thank Dr Tomomi Gotoh (Kumamoto University, Kumamoto, Japan) for providing us with CHOP-deficient mice.

### Sources of Funding

This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (No. 17590731) and a grant from the Japan Cardiovascular Research Foundation (No. 19390220).

### Disclosures

None.

### References


**CLINICAL PERSPECTIVE**

Heart failure is a major and growing public health problem worldwide. Although cardiac hypertrophy is a risk factor for the development of heart failure, it is largely unknown how prolonged cardiac hypertrophy causes heart failure. Recently, accumulating evidence has demonstrated that a number of diseases, including neurodegenerative diseases and diabetes mellitus, are associated with the impairment of protein folding in the endoplasmic reticulum (ER). The ER responds to stress by upregulating ER chaperones or attenuating global protein synthesis, but prolonged and/or excess ER stress leads to apoptosis. Here, we provide evidence that C/EBP homologous protein (CHOP), a transcriptional factor that mediates ER-initiated apoptotic cell death, and the ER chaperone were elevated in human failing heart samples, suggesting that ER stress is induced in human failing hearts. Pressure overload induced cardiac hypertrophy and failure, along with increased expression in the ER chaperone and CHOP in mice heart. Interestingly, CHOP-deficient mice showed less cardiac hypertrophy and better cardiac function after pressure overload. One possible mechanism for reduced cardiac hypertrophy was enhanced phosphorylation of eukaryotic translation initiation factor 2alpha, which reduces protein translation and is negatively regulated by CHOP, in pressure-overloaded hearts of CHOP-deficient mice. Furthermore, CHOP decreased Bcl2 protein levels and other Bcl2 family members in cardiomyocytes, suggesting that the ER-mitochondria pathway would play an important role in cell death in pressure-overloaded hearts. In conclusion, the present findings suggest that CHOP may be a logical target for development of drugs to prevent cardiac hypertrophy and cardiomyocyte cell death in failing hearts.
Ablation of C/EBP Homologous Protein Attenuates Endoplasmic Reticulum–Mediated Apoptosis and Cardiac Dysfunction Induced by Pressure Overload
Hai Ying Fu, Ken-ichiro Okada, Yulin Liao, Osamu Tsukamoto, Tadashi Isomura, Mitsutoshi Asai, Tamaki Sawada, Keiji Okuda, Yoshihiro Asano, Shoji Sanada, Hiroshi Asanuma, Masanori Asakura, Seiji Takashima, Issei Komuro, Masafumi Kitakaze and Tetsuo Minamino

Circulation. 2010;122:361-369; originally published online July 12, 2010;
doi: 10.1161/CIRCULATIONAHA.109.917914

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SUPPLEMENTARY MATERIAL

Supplementary methods:

Adult cardiomyocytes were isolated from 8-week-old WT or CHOP knockout mice and were stored in Hanks buffer (Invitrogen Corp.) containing 1.2 mmol/L CaCl₂. Field stimulation at 1 Hz pacing rate was done with 5 ms square pulses of constant voltage at 20% above threshold. The contraction of cardiomyocyte was recorded with an inverted microscopy (Olympus IX81, Olympus Corp. Tokyo).

Movie Legend:

Movies 1 and 2 are the videos of the contractions of adult cardiomyocytes from WT and CHOP-deficient mouse, respectively. Best viewed with Windows Media Player.