Prolonged Endoplasmic Reticulum Stress in Hypertrophic and Failing Heart After Aortic Constriction
Possible Contribution of Endoplasmic Reticulum Stress to Cardiac Myocyte Apoptosis

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Background—The endoplasmic reticulum (ER) is recognized as an organelle that participates in folding secretory and membrane proteins. The ER responds to stress by upregulating ER chaperones, but prolonged and/or excess ER stress leads to apoptosis. However, the potential role of ER stress in pathophysiological hearts remains unclear.

Methods and Results—Mice were subjected to transverse aortic constriction (TAC) or sham operation. Echocardiographic analysis demonstrated that mice 1 and 4 weeks after TAC had cardiac hypertrophy and failure, respectively. Cardiac expression of ER chaperones was significantly increased 1 and 4 weeks after TAC, indicating that pressure overload by TAC induced prolonged ER stress. In addition, the number of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL)–positive cells increased, and caspase-3 was cleaved in failing hearts. The antagonism of angiotensin II type 1 receptor prevented upregulation of ER chaperones and apoptosis in failing hearts. On the other hand, angiotensin II upregulated ER chaperones and induced apoptosis in cultured adult rat cardiac myocytes. We also investigated possible signaling pathways for ER-initiated apoptosis. The CHOP- (a transcription factor induced by ER stress), but not JNK- or caspase-12–, dependent pathway was activated in failing hearts by TAC. Pharmacological ER stress inducers upregulated ER chaperones and induced apoptosis in cultured cardiac myocytes. Finally, mRNA levels of ER chaperones were markedly increased in failing hearts of patients with elevated brain natriuretic peptide levels.

Conclusions—These findings suggest that pressure overload by TAC induces prolonged ER stress, which may contribute to cardiac myocyte apoptosis during progression from cardiac hypertrophy to failure. (Circulation. 2004;110:705-712.)

Key Words: apoptosis ■ hypertrophy ■ heart failure ■ endoplasmic reticulum
regulated protein 94 kDa (GRP94), GRP78, and calreticulin. When ER stress is excess and/or prolonged, however, the initiation of the apoptotic processes is promoted by transcriptional induction of C/EBP homologous protein (CHOP) or by the activation of c-JUN NH2-terminal kinase (JNK)– and/or caspase-12–dependent pathways. Accumulating evidence demonstrates that apoptosis initiated by the ER is involved in the pathogenesis of neurodegenerative diseases and diabetes mellitus. Interestingly, apoptosis is also the key contributor to cell loss in the setting of heart failure. Because the development of the ER and elevated protein synthesis in hearts suggest that ER stress is induced in hypertrophic and failing hearts, we hypothesized that ER stress may contribute to cardiac myocyte apoptosis observed during progression from cardiac hypertrophy to failure. To test this hypothesis, we examined the potential role of ER stress–induced apoptosis in experimental models and failing human hearts. Furthermore, because the renin–angiotensin system plays an important role in the development of hypertrophic and failing hearts and angiotensin II increases protein synthesis that may potentially cause ER stress, we also examined the role of angiotensin II in the induction of ER stress in pathophysiological hearts and cultured cardiac myocytes.

Methods

Antibodies and Reagents
Tunicamycin, thapsigargin, and angiotensin II were purchased from Sigma. Antibodies for GRP78, GRP94, calreticulin, and KDEL (Lys-Asp-Glu-Leu), which recognizes both GRP78 and GRP94, were purchased from StressGen. Antibodies for CHOP and actin were obtained from Santa Cruz Biotechnology. Antibodies for phospho-SAPK/JNK, SAPK/JNK, and cleaved caspase-3 were obtained from Cell Signaling Technology. The antibody for caspase-12 was obtained from BioVision. CS-866, an angiotensin II type 1 receptor blocker, and RNH-6270, an active metabolite of CS-866, were provided from Sankyo Co Ltd.

Preparation of Rat Cardiac Myocytes
Primary cultures of neonatal (aged 2 to 3 days) and adult (aged 7 weeks; male) cardiac myocytes were prepared with the use of Wistar-Kyoto rats as described previously. All procedures were performed 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.

Immunoblotting
Immunoblotting was performed as described previously, and immunoreactive bands were quantified by densitometry (Molecular Dynamics).

Preparation of Human Heart Samples
Under the protocol approved by the institutional review board of the National Cardiovascular Center (No. 14-18), samples of human cardiac tissue were obtained. Tissue samples for RNA extraction were frozen at −80°C until use, whereas the specimens for immunohistochemistry were fixed and embedded in paraffin.

Echocardiographic Data

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=7)</th>
<th>TAC, 1 Week (n=9)</th>
<th>TAC, 4 Weeks (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventricular septum, mm</td>
<td>0.52±0.01</td>
<td>0.81±0.02*</td>
<td>0.99±0.01*</td>
</tr>
<tr>
<td>Posterior wall, mm</td>
<td>0.53±0.01</td>
<td>0.75±0.01*</td>
<td>0.97±0.02*</td>
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<tr>
<td>Left ventricular end-diastolic dimension, mm</td>
<td>2.85±0.05</td>
<td>2.84±0.09</td>
<td>3.15±0.06*</td>
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<tr>
<td>Left ventricular end-systolic dimension, mm</td>
<td>1.52±0.07</td>
<td>1.50±0.05</td>
<td>1.89±0.04*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>46.4±1.8</td>
<td>47.2±1.0</td>
<td>40.0±1.0*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Fractional shortening was calculated as (left ventricular end-diastolic dimension − left ventricular end-systolic dimension) / left ventricular end-diastolic dimension.

*P<0.05 vs sham operation.
Northern Blot Analysis
Northern blotting was performed as described previously. To synthesize specific probes for Northern blotting, we used the following sense and antisense primers: 5'-GCCACCGGGATGGTTCCTTGCC-3' and 5'-GCGGATCCAGGTCGACGCCGGCCA-3' for GRP78 (398 bp); 5'-CTGTGGGGGCGGCTATGTGA-3' and 5'-CACCCCAAATCCGAACCAGC-3' for calreticulin (1069 bp); 5'-GCAATTCCACCTAGGTCA-3' and 5'-GGCTCCAATCTGTCATCC-3' for ANP (375 bp); 5'-AGTCCCTCCAGAGACATG-3' and 5'-CTTGTGGAATCAGAAGCA-3' for BNP (462 bp); and 5'-TCACCATCTTCCAGGAGCGA-3' and 5'-TCCTTGGAGGCCATGTGGGC-3' for GAPDH (784 bp), respectively. As the normal controls, we used total human heart RNA purchased from BD Bioscience and BioChain.

Immunohistochemical Analysis
Immunohistochemistry was performed as described previously. TAC Model
C57BL/6 mice (aged 8 weeks; male) were subjected to TAC or sham operation as described previously. Echocardiography was performed on the mice as described previously. CS-866 (1 mg/kg per day) was administered by gavage 1 day after TAC operation.

Apoptotic Cell Assay
Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) reaction was performed with the use of ApoTag apoptosis detection kits (Intergen Company). DNA ladder was performed as described previously. Double staining for TUNEL and desmin (DakoCytomation) was performed in the left ventricular (LV) free wall of mice. Neonatal rat cardiac myocytes were treated with tunicamycin (0.1 μg/mL) or thapsigargin (0.1 μmol/L) for 48 hours and stained for TUNEL and sarcomeric myosin heavy chain (MF-20) (Hybridoma Bank, University of Iowa). Numbers of TUNEL-positive cells counted in desmin-positive cells (n=5000) and in MF-20-positive cells (n=500) are expressed as percentages.

MTT Assay
Cardiac myocytes were treated with tunicamycin or thapsigargin for 48 hours. Cell viability was assessed by MTT assay according to the manufacturer’s instructions (Cell Counting Kit 8). The relative number of viable cells was determined by estimating the value for untreated cells as 100%.

Statistical Analysis
Data are expressed as mean±SEM. Results were compared by 1-way ANOVA followed by Bonferroni’s test. Comparisons of categorical variables were generated by Fisher’s exact test. P<0.05 was accepted as statistically significant.

Results
Cardiac Hypertrophy and Failure in Mice After TAC
One week after the onset of TAC, cardiac enlargement was detected without severe lung congestion (Figure 1A, 1B). In contrast, cardiac enlargement was more prominent along with marked lung congestion 4 weeks after TAC (Figure 1A, 1B). Echocardiographic analysis also revealed LV dilatation and...
LV systolic dysfunction 4 weeks, but not 1 week, after TAC. Increases in LV wall thickness were found 1 week after TAC and thereafter (Table, Figure 1C). These findings indicate that the mice 1 and 4 weeks after surgery corresponded to models of cardiac hypertrophy and failure, respectively.

**Upregulation of ER Chaperones in Hypertrophic and Failing Mouse Hearts**

mRNA levels of ER chaperones including GRP78 and calretilculin and of ANP were increased by TAC (Figure 2A). Consistent with mRNA levels, protein levels of ER chaperones were increased in hearts after TAC (Figure 2B). Moreover, immunohistochemical analysis revealed that the number of KDEL-positive cells was increased in the hearts of the mice after TAC (Figure 2C). Furthermore, the number of TUNEL-positive cells was increased in the heart 4 weeks after TAC (Figure 2D). Next, we checked involvement of signaling pathways of ER-initiated apoptosis in the hearts after TAC. CHOP was markedly induced 4 weeks after TAC, which coincided with the timing of the appearance of apoptosis (Figure 2E). The caspase-12 cleavage or JNK phosphorylation was not observed in hearts after TAC (Figure 2E, 2F).

**Prevention of ER Chaperone Expression by Antagonism of Angiotensin II Type 1 Receptor in Mouse Hearts After TAC**

The administration of CS-866 markedly attenuated the induction of both ER chaperones and ANP (Figure 3A). Furthermore, this angiotensin II type 1 receptor blocker prevented the development of cardiac hypertrophy and failure (Figure 3B), decreased the number of TUNEL-positive cells (Figure 3C), and attenuated the cleavage of caspase-3 in the failing hearts (Figure 3D). CS-866 attenuated CHOP induction but did not affect phosphorylation of JNK or the cleavage of caspase-12 in the failing hearts (Figure 3E).

**Upregulation of ER Chaperones and Apoptosis by Angiotensin II in Adult Rat Cardiac Myocytes**

Using adult rat cardiac myocytes, we studied the effects of angiotensin II on the expression of ER chaperones and apoptosis (Figure 4A). Treatment of adult rat cardiac myocytes with angiotensin II (10^{-7} mol/L) for 24 hours induced cardiac myocyte apoptosis along with the inductions of ER chaperones and CHOP, either of which was inhibited by RNH-6270 (10^{-7} mol/L) (Figure 4B). Angiotensin II also increased protein synthesis evaluated by incorporation of [3 H]leucine, which was prevented by RNH-6270 (data not shown). Quantitative analysis revealed that angiotensin II increased KDEL-positive cells compared with no treatment (5.0±0.1% vs 16.0±1.1%; P<0.05; n=6000 each). The prevalence of TUNEL-positive cells in KDEL-positive ones was significantly higher than that in KDEL-negative ones (P<0.05, Fisher’s exact test) (Figure 4B, 4C), indicating the association between ER stress and cardiac apoptosis. Furthermore, we could not find any KDEL-negative cells among...
CHOP-positive ones, suggesting that CHOP specifically mediated ER-initiated signaling (Figure 4C). Importantly, there was a high prevalence of CHOP-positive cells among TUNEL-positive cells (69.7%) (P<0.05, Fisher’s exact test), suggesting that CHOP-mediated signaling contributes to angiotensin II–induced cardiac apoptosis. RNH-6270 attenuated the upregulation of ER chaperones, induction of CHOP, or cardiac myocyte apoptosis (data not shown).

Apoptosis of Cultured Cardiac Myocytes Induced by Pharmacological Agents That Impair ER Function

Tunicamycin or thapsigargin, either of which impairs ER function, increased protein levels of ER chaperones in cultured cardiac myocytes (Figure 5A). Treatment of cardiac myocytes with tunicamycin or thapsigargin caused a decrease of cell viability (Figure 5B) and an increase of TUNEL-positive cells in a dose-dependent manner (Figure 5C, 5D). Pharmacological ER stress inducers caused DNA fragmentation and the cleavage of caspase-3 (Figure 5E and 5F), and they induced CHOP expression, caspase-12 cleavage, and JNK phosphorylation in cultured cardiac myocytes (data not shown). Immunohistochemical analysis revealed that tunicamycin increased KDEL-positive cells compared with no treatment (31.6% versus 73.5%; P<0.05; n=500 each). The prevalence of TUNEL-positive cells in KDEL-positive ones was significantly higher than that in KDEL-negative ones (P<0.05, Fisher’s exact test) (Figure 5G and 5H), suggesting the association between ER stress and cardiac myocyte apoptosis.

Expression of ER Chaperones in Failing Human Hearts

In hematoxylin-eosin staining of cardiac tissue from patients with dilated cardiomyopathy, vacuolation was prominent, as previously reported. Immunohistochemical analysis revealed that ER chaperones, including GRP78/GRP94 and calreticulin, were induced in the hearts of 2 patients with dilated cardiomyopathy (Figure 6A, d through f and g through i) but not in the hearts of control subjects (Figure 6A, a through c). These changes were also found in adriamycin-induced cardiomyopathy, in which the development of the ER was reported previously (Figure 6A, j through l). We confirmed that areas in which ER chaperones were induced corresponded to vacuolation areas in hematoxylin-eosin staining (Figure 6A). We also examined the levels of mRNA for ER chaperones in the hearts of 2 control subjects (Figure 6B, lanes 1, 2) and 8 patients with dilated cardiomyopathy (Figure 6B, lanes 3 through 10). Consistent with the clinical diagnosis, mRNA levels of ANP and BNP were increased in the hearts of all 8 patients with dilated cardiomyopathy (lanes 3 through 10) but not in the hearts of control subjects (Figure 5A).
In 7 of the 8 patients with dilated cardiomyopathy (lanes 3 through 10), expression of both GRP78 and calreticulin was also markedly induced compared with the control subjects (Figure 6B).

**Discussion**

The ER is one of the largest cell organelles, and the ER lumen and internal spaces constitute >10% of the entire cell volume. The functional and genomic analyses have revealed that the genes upregulated by ER stress account for 6% of the yeast genome. These findings indicate that the ER is a highly dynamic organelle and plays essential roles in cell viability. The ER participates in folding of secretory and membrane proteins. When the ER is overloaded to deal with the enhanced synthesis of secretory proteins, molecular compensatory mechanisms will operate (1) upregulation of ER chaperones, (2) reduced translation that decreases the load of new protein synthesis, and (3) degradation of proteins misfolded in the ER.

In the present study, we evaluated the upregulation of ER chaperones as markers of ER stress. In mice with hypertrophic and failing hearts induced by TAC, we confirmed that ER chaperones were markedly induced. These findings suggest that pressure overload by TAC induces prolonged ER stress in the heart. The ER in the heart contains a high density of Ca\(^{2+}\)-ATPase and is often referred to as the sarcoplasmic reticulum.

We demonstrated that CS-866, an angiotensin II type 1 receptor blocker, attenuated the upregulation of ER chaperones, the development of cardiac hypertrophy and failure, and cardiac myocyte apoptosis in TAC hearts. Furthermore, treatment of adult cardiac myocytes with angiotensin II induced ER chaperones as well as protein synthesis, either of which was prevented by RNH-6270. These findings suggest that cardiac ER stress is induced via an angiotensin II type 1 receptor-dependent pathway. Although it is likely that one of the potential mechanisms by which angiotensin II induced ER stress is enhanced protein synthesis, further investigation about the intracellular signaling pathway by which angiotensin II induces ER stress will be needed.
Recent studies have demonstrated the occurrence of organelle-specific initiation of cell death. Upregulation of ER chaperones was found in the heart from 1 week after TAC, suggesting the possibility that the ER initiates cardiac myocyte apoptosis. However, TUNEL-positive cells were not seen at 1 week after TAC and only became apparent at 4 weeks after TAC. If apoptosis in failing hearts was due to ER stress, time courses of the onsets of ER stress and cardiac apoptosis/dysfunction were inconsistent. This discrepancy between ER stress and the delayed appearance of pathological changes is also found in PERK-deficient mice that cannot reduce the initiation of translation in response to ER stress. Prolonged ER stress is needed to impair the heart function that precedes metabolic decompensation in PERK-deficient mice. In the clinical setting, presenilin-1 gene mutation linked to early-onset familial Alzheimer’s disease is known to downregulate the unfolded protein response and increase vulnerability to ER stress. Usually, dementia due to presenilin-1 gene mutation appears in the 40s. These findings suggest that prolonged ER stress would be essential to cause overt pathological conditions.

ER stress induces apoptosis through CHOP-, JNK-, and caspase-12-dependent signaling pathways. The overexpression of CHOP leads to growth arrest and apoptosis. Thus, the induction of CHOP indicates that the ER-initiated apoptosis is promoted. Caspase-12 is located on the ER membrane and is activated only by ER stress. JNK belongs to the stress-activated protein kinases and has been shown to induce apoptosis in response to ER stress. We demonstrated that the number of TUNEL-positive cardiac myocytes was significantly increased in mouse hearts 4 weeks after TAC, and CHOP, but not JNK or caspase-12, was simultaneously induced. Because CHOP is a transcription factor that specifically mediates ER-initiated apoptosis, the induction of CHOP in failing hearts after TAC indicates that ER-initiated apoptosis is promoted under these conditions. However, because these signaling pathways were activated in response to ER stress inducers in cultured rat cardiac myocytes, we cannot exclude the possibility that signaling pathways other than CHOP are activated at different timings.

We used tunicamycin or thapsigargin as a pharmacological ER stress inducer. Thapsigargin is the ER Ca\(^{2+}\)-ATPase inhibitor that leads to ER stress.
tunicamycin is a specific inhibitor of N-glycosylation, a process that is observed only in the ER, indicating that this agent is highly specific ER stress inducer.\textsuperscript{13,15} Immunohistochemical analysis revealed the association between KDEL-positive cells and apoptotic cells when neonatal rat cardiac myocytes were treated with tunicamycin, supporting the idea that ER stress can initiate apoptotic signaling in cardiac myocytes. The difference in number of KDEL-positive cells in neonatal and adult cardiac myocytes is probably due to the differences in protein synthetic capacity and/or the presence of fetal calf serum in cultured medium.

The heart could become a neuroendocrine organ in the setting of chronic heart failure.\textsuperscript{5} We found that both the ER expansion and the upregulation of ER chaperones were found in patients with heart failure. These changes suggestive of ER stress in human chronic heart failure may be attributed to prolonged elevation of protein synthesis such as ANP and BNP. These findings suggest that ER-initiated apoptosis due to prolonged ER stress in failing human hearts may also be involved in the development and progression of clinical heart failure.\textsuperscript{14,15}

In conclusion, to our knowledge, the present study is the first demonstration of ER stress in hypertrophic and failing hearts under experimental and clinical conditions. Our findings suggest that ER-initiated apoptosis may contribute to cardiac myocyte apoptosis in failing hearts.

Acknowledgments

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References

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