Increased Endoplasmic Reticulum Stress in Atherosclerotic Plaques Associated With Acute Coronary Syndrome

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Background—The endoplasmic reticulum (ER) responds to various stresses by upregulation of ER chaperones, but prolonged ER stress eventually causes apoptosis. Although apoptosis is considered to be essential for the progression and rupture of atherosclerotic plaques, the influence of ER stress and apoptosis on rupture of unstable coronary plaques remains unclear.

Methods and Results—Coronary artery segments were obtained at autopsy from 71 patients, and atherectomy specimens were obtained from 40 patients. Smooth muscle cells and macrophages in the fibrous caps of thin-cap atheroma and ruptured plaques, but not in the fibrous caps of thick-cap atheroma and fibrous plaques, showed a marked increase of ER chaperone expression and apoptotic cells. ER chaperones also showed higher expression in atherectomy specimens from patients with unstable angina pectoris than in specimens from those with stable angina. Expression of 7-ketocholesterol was increased in the fibrous caps of thin-cap atheroma compared with thick-cap atheroma. Treatment of cultured coronary artery smooth muscle cells or THP-1 cells with 7-ketocholesterol induced upregulation of ER chaperones and apoptosis, whereas these changes were prevented by antioxidants. We also investigated possible signaling pathways for ER-initiated apoptosis and found that the CHOP (a transcription factor induced by ER stress)-dependent pathway was activated in unstable plaques. In addition, knockdown of CHOP expression by small interfering RNA decreased ER stress-dependent death of cultured coronary artery smooth muscle cells and THP-1 cells.

Conclusions—Increased ER stress occurs in unstable plaques. Our findings suggest that ER stress-induced apoptosis of smooth muscle cells and macrophages may contribute to plaque vulnerability.

Key Words: apoptosis • plaque • myocardial infarction • endoplasmic reticulum

Most of the acute clinical manifestations of coronary atherosclerosis result from plaque rupture that triggers thrombosis and vessel occlusion, producing the acute coronary syndrome (ACS).1-3 Previous reports have shown that apoptosis affects all of the types of cells residing within atherosclerotic plaques, including smooth muscle cells (SMCs) and macrophages,4,5 with oxidized low-density lipoprotein and several inflammatory factors being known to induce apoptosis.6,7 The number of apoptotic cells depends on the plaque stage and is generally higher in more advanced plaques.6,8 SMCs synthesize most of the interstitial collagen that stabilizes the fibrous cap of a plaque.4,7 Therefore, excessive apoptosis of SMCs in the fibrous cap may compromise plaque integrity and render it vulnerable to proteolytic attack by inflammatory cells, leading to plaque rupture.4,7 Apoptotic macrophages are more frequent at sites of plaque rupture than in areas where the fibrous cap remains intact.9 A decrease in macrophages would reduce the scavenging of apoptotic SMCs and macrophages, allowing the cells to undergo secondary necrosis, thereby increasing thrombogenicity of the plaque.10

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The online-only Data Supplement, consisting of expanded Methods, tables, and figures, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.106.682054/DC1.

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including ischemia, hypoxia, heat shock, mutation, increased protein synthesis, and reactive oxygen species, all of which can potentially lead to ER dysfunction. \textsuperscript{11,12} In response to ER stress, there is marked upregulation of various ER chaperones, such as the 94-kDa glucose-regulated protein (GRP94) or GRP78 that stabilizes protein folding. \textsuperscript{11,13,14} When the ER becomes overloaded with misfolded proteins, the unfolded protein response (UPR) occurs to enhance cell survival.\textsuperscript{15} However, prolonged ER stress can trigger apoptotic cell death, which is promoted by transcriptional induction of C/EBP homologous protein (CHOP) and/or by the activation of c-JUN NH\textsubscript{2}-terminal kinase (JNK)– and/or caspase-12–dependent pathways.\textsuperscript{16} In support of this concept, our investigation of the effects of prolonged ER stress on hypertrophic and failing hearts revealed that apoptosis of cardiac myocytes was induced via activation of CHOP, an ER-specific proapoptotic factor.\textsuperscript{17} An important role of ER-initiated cell death pathways has also been demonstrated in several diseases, including diabetes mellitus,\textsuperscript{16} neurodegenerative conditions,\textsuperscript{18} and ischemia.\textsuperscript{19}

Oxidation of low-density lipoprotein plays a significant pathogenetic role in atherosclerosis.\textsuperscript{6,7,20} In cultured peritoneal macrophages, excessive accumulation of free cholesterol (induced by acetyl low-density lipoprotein with an acyl-CoA:cholesterol acyltransferase [ACAT] inhibitor) initiates ER stress, increases CHOP expression, and leads to apoptosis.\textsuperscript{21} Studies of apoE\textsuperscript{-/-} mice also support the relevance of ER stress to macrophage apoptosis and to enlargement of the necrotic core in advanced atherosclerotic plaques.\textsuperscript{22,23} However, it is still unclear whether ER stress and UPR activation have a role in plaque rupture. Unfortunately, the absence of a suitable animal model has greatly hindered investigation of the molecular mechanisms of plaque rupture and evaluation of the effects of ER stress in vivo.\textsuperscript{24,25}

In the present study, we examined histological sections from atherosclerotic coronary artery lesions obtained at autopsy or after directional coronary atherectomy (DCA) to investigate markers of ER stress/UPR activation and apoptotic cell death. Oxysterols such as 7-ketocholesterol (7-KC) have been reported to be partially responsible for the cytotoxicity of oxidized low-density lipoprotein.\textsuperscript{26,27} Exposure of cultured human SMCs to 7-KC induces the UPR and promotes apoptotic cell death.\textsuperscript{28} So we investigated 7-KC expression in plaque specimens by immunohistochemistry. We also examined whether 7-KC could activate ER stress using cultured human coronary artery SMCs (CASMCs) and a monocyte cell line (THP-1). Furthermore, we investigated the possible signaling pathways for ER-initiated apoptosis, and we found that the CHOP (a transcription factor induced by ER stress)-dependent pathway was activated in unstable plaques, whereas knockdown of CHOP expression by small interfering RNA (siRNA) decreased ER stress-dependent death of cultured CASMCs and THP-1 cells.

**Methods**

**Coronary Artery Specimens**

Two different sets of specimens were obtained under a protocol approved by the Institutional Review Board of the National Cardiovascular Center and Miyazaki University. The first set of specimens was obtained at autopsy, and the second set was obtained by DCA. Classification of the histology of the lesions in autopsy specimens was done morphologically, as described previously (Table 1).\textsuperscript{29–31} Demographic data for the study population are presented in Table I of the Data Supplement.

In brief, 152 coronary artery segments were obtained at autopsy from 71 patients, including 17 consecutive patients who experienced fatal ACS without percutaneous coronary intervention and 54 consecutive patients with noncardiac death. The major coronary arteries and their branches were cut transversely at \( \pm 5 \)-mm intervals, and 17 ruptured plaques were detected in the 17 ACS patients (ruptured: AHA type VI, \( n = 17 \)). The remaining 33 patients with noncardiac death and the 17 ACS patients also had advanced unruptured plaques (\( \geq 75\% \) cross-sectional luminal narrowing), and we assessed each segment at the narrowest point (\( n = 114 \)). The advanced atherosclerotic unruptured plaques were additionally divided into fibrous plaques (fibrous: fibrocellular tissue was the predominant component, and the lipid core was inconspicuous or absent; AHA type Vc, \( n = 48 \)), thick-cap atheroma (thick: a lipid core covered by a fibrous cap \( > 65-\mu \text{m} \) thick; AHA type Va, \( n = 51 \)), and thin-cap atheroma (thin: a lipid-rich core covered by a fibrous cap \( < 65-\mu \text{m} \) thick; AHA type Va, \( n = 15 \)). Another 21 patients with noncardiac death who had no advanced unruptured plaques and normal coronary arteries that only showed diffuse intimal thickening (normal: AHA type I, \( n = 21 \)) were used as a control group.

We performed a morphological analysis of multiple lesions (\( n = 152 \)) obtained at autopsy from 71 patients (Figure 1). The supplementary analyses included pairwise comparison of unruptured and ruptured plaques from each heart of each patient with ACS (Data Supplement Figure I) and investigation of the correlation between traditional cardiovascular risk factors and ER stress (Data Supplement Table II and Figure III). These supplementary analyses were based on representative data from each patient. (Details of the methods used to perform the supplementary analyses are included in the expanded Methods section in the Data Supplement.)

Forty DCA specimens were obtained from 40 patients who were treated for stable angina pectoris (SAP; \( n = 20 \)) or unstable angina pectoris (UAP; \( n = 20 \)). One DCA specimen was obtained per patient, and these specimens were classified on the basis of the clinical situation at the time of DCA (Table 2). These specimens were fixed in 4% paraformaldehyde for 6 hours at 4°C and then embedded in paraffin.

**Immunohistochemistry**

Serial sections were examined by immunohistochemistry, as described previously.\textsuperscript{17} In brief, sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 0.3% \( \text{H}_2\text{O}_2 \) in methanol for 30 minutes. For some antibodies, antigen retrieval was performed as specified below. After blocking with 3% normal bovine serum albumin, sections were incubated with the primary antibody overnight at 4°C. KDEL (Lys-Asp-Glu-Leu) antibody, which recognizes both GRP78 and GRP94, was purchased from Stressgen (San Diego, Calif) and was used at a dilution of 1:2000. Anti-CHOP antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif) and was applied at a dilution of 1:600.
after antigen retrieval by incubation for 10 minutes at room temperature in 5 μg/mL proteinase K. Anti-phospho-c-JUN NH2-terminal kinase antibody was used to detect c-JUN kinase (JNK), which is involved in the UPR.16 It was obtained from Cell Signaling (Danvers, Mass) and was applied at a dilution of 1:100 after heat retrieval for 15 minutes at a sub-boiling temperature in 1 mmol/L EDTA (pH 8.0). Colon carcinoma sections were stained with anti-phospho-JNK antibody after preincubation with each synthetic peptide used for immunization (KDEL: synthetic peptide SEKDEL, 10 μg/mL, Tore Bio, CHOP peptide: 10 μg/mL, Santa Cruz Biotechnology) resulted in no detectable signals, demonstrating the specificity of the antibody (Data Supplement Figure II).

**TABLE 2. Human Coronary Specimens (Atherectomy; n=40)**

<table>
<thead>
<tr>
<th>Origin and Classification of Plaques</th>
<th>No. of Specimens</th>
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<tbody>
<tr>
<td>SAP</td>
<td>20</td>
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<tr>
<td>UAP</td>
<td>20</td>
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at a dilution of 1:200. The EnVision kit (DAKO) was then used for immunostaining. Application of the KDEL antibody or the CHOP antibody after preincubation with each synthetic peptide used for immunization (KDEL: synthetic peptide SEKDEL, 10 μg/mL, Tore Bio, CHOP peptide: 10 μg/mL, Santa Cruz Biotechnology) resulted in no detectable signals, demonstrating the specificity of the antibody (Data Supplement Figure II).

**Terminal dUTP Nick End-Labeling Method and Double Immunohistochemistry**

Cells undergoing apoptosis were identified by the terminal dUTP nick-end-labeling (TUNEL) method with the ApopTag In Situ Apoptosis Detection Kit (Chemicon, Temecula, Calif), as described previously.8 For simultaneous identification of CHOP and TUNEL immunoreactivity, double immunostaining of specimens was performed. First, the TUNEL method was performed with an ApopTag...
Kit, and then CHOP was detected with an alkaline phosphatase–labeled secondary antibody with NewFukusin (DAKO).

**In Situ Hybridization**

Digoxigenin-labeled cRNA probes and the negative control (LNE120) were purchased from Direct Communications Inc (Hiro-saki, Japan), and the sequences were as follows: GRP78: 5′-UOGAAGUCGUGGCGUCACCAAAACAGCAUGU-UCAUUCGUUCUUCUUCUAUU-CAGCUAGGUCGUCACCCAAUCUCCCUGGUCAGGCGCUCGAUUUC-CAUGUGCAGAUUCACCAUUCGGUCAAUCAGAGCUCGG-AUGCUCCCAAUUGUUCAUG-

**Results**

**Upregulation of ER Chaperones and Apoptosis in the Fibrous Caps of Thin-Cap Atheroma and Ruptured Plaques**

In the fibrous caps of thin-cap atheroma and ruptured plaques, KDEL and CHOP immunostaining showed a marked increase compared with the level of staining in the fibrous caps of thick-cap atheroma and fibrous plaques (Figure 1A k through t, Figure 1B, and Figure 1C). KDEL-positive cells were more numerous than CHOP-positive cells in the fibrous caps of thin-cap atheroma and ruptured plaques. Most of the CHOP-positive cells also expressed KDEL, as shown by staining of serial sections. In the same hearts of the ACS patients, there was a significant difference of KDEL- and CHOP-positive cells between the unruptured and ruptured plaques (Data Supplement Figure I). We also assessed ER chaperone (GRP78) and CHOP expression at the mRNA level by ISH (Figure 1D). Furthermore, we confirmed that the KDEL- and CHOP-positive cells were SMCs or macrophages by immunostaining of serial sections with anti α-smooth muscle actin and anti-CD68, respectively (Figure 1A, u through δ). In the fibrous caps of thin-cap atheroma and ruptured plaques, colocalization of CHOP immunoreactivity with TUNEL-positive cells was observed by double immunostaining (Figure 1E). The percentage of TUNEL-positive cells (Figure 1F) and the percentage of TUNEL-positive cells among CHOP-positive cells (Figure 1G) were increased compared with the findings in other specimens. Immunostaining for phospho-JNK, which is a proapoptotic factor involved in ER stress, revealed no immunoreactivity in the fibrous caps of thick, thin, or ruptured plaques (data not shown). In normal coronary artery specimens with diffuse intimal thickening (Figure 1A, k and p), there was no KDEL or CHOP positivity. In the region around the necrotic core of advanced plaques, KDEL positivity was only observed in macrophages. There was no significant difference in the number of KDEL-positive cells within the area surrounding the necrotic core of thick-cap atheromas (726±88/mm²), thin-cap atheromas (741±52/mm²), and ruptured plaques (651±102/mm²).

**Immunohistochemical Detection of 7-KC in the Fibrous Caps of Atherosclerotic Plaques**

To explore the likely molecular mechanism of activation of ER stress and the mechanistic link to apoptosis, we investigated plaque lipids by staining frozen coronary artery sections with anti-7-KC antibody (Figure 3), and the in vitro studies were performed (Figure 4). Immunoreactivity for 7-KC was increased in the fibrous caps of thin-cap atheroma, whereas no immunoreactivity was detected in the fibrous caps of thick-cap atheroma (Figure 3). In the region around the lipid core, however, 7-KC immunoreactivity was visible in both types of atheroma (Figure 3).

**Upregulation of ER Chaperones, CHOP, and Apoptosis by 7-KC and Effects of CHOP Knockdown by siRNA in CASMCs or THP-1 Cells**

Exposure of cells to 7-KC increased the expression of GRP78 and CHOP mRNA, whereas this increase was prevented by
the antioxidants N-acetylcysteine or glutathione (Figure 4A). We observed intracellular production of reactive oxygen species after exposure to 7-KC, whereas glutathione reduced reactive oxygen species production (Figure 4B). We also examined the effects of 7-KC on apoptosis of CASMCs and THP-1 cells (Figure 4C). Treatment with 7-KC increased FITC-annexin and propidium iodide staining in a dose-dependent (Figure 4C, b and c) and time-dependent (data not shown) manner. Treatment of CASMCs and THP-1 cells with 7-KC for 24 hours also induced apoptosis along with the induction of ER chaperones and CHOP at the protein level (Figure 4D). When CASMCs and THP-1 cells were simultaneously incubated with 7-KC and N-acetylcysteine or glutathione, both antioxidants reduced the induction of ER chaperones (Figure 4D). Quantitative analysis revealed that most of the CHOP-positive cells coexpressed KDEL (88.2% of CHOP-positive CASMCs and 72.7% of CHOP-positive THP-1 cells; P < 0.05, Fisher’s exact test), whereas there were few KDEL-negative and CHOP-positive cells, which suggests that CHOP was involved in the mediation of ER-initiated signaling (Figure 4D, c and d). Treatment of THP-1 cells with 7-KC induced CHOP, whereas 2 different siRNAs targeting CHOP caused the knockdown of CHOP expression (Figure 4E, a). Knockdown of CHOP expression by siRNA decreased the number of TUNEL-positive THP-1 cells after exposure to 7-KC (Figure 4E, b and d). Similarly, the knockdown of CHOP expression by siRNA decreased the number of TUNEL-positive CASMCs after exposure to 7-KC (Figure 4E, c).
Discussion

The present study revealed a marked increase of ER chaperone expression, CHOP expression, and apoptosis in the fibrous caps of thin-cap atheroma or ruptured plaques, as well as in atherectomy specimens from UAP patients, which suggests that ER stress may play a role in the progression of plaque vulnerability and the occurrence of acute complications of coronary atherosclerosis in humans. Because of the inherent limitations of an autopsy study, we could not exclude the possibility that UPR activation occurred after plaque rupture. Previous reports have shown that ER chaperones, such as GRP78 or GRP94, may have a protective effect against ischemia/reperfusion injury. However, the presence of apoptotic changes in the thin-cap atheroma may be one of the reasons why noncardiac death suggested that the findings we observed in ruptured plaques represented the evolution of such changes in the thin-cap atheroma rather than being secondary to plaque rupture or ischemia/reperfusion injury. Only specimens from patients without percutaneous coronary intervention were studied, to exclude the influence of this intervention. We also observed an increase of ER stress-related changes in freshly fixed atherectomy specimens obtained from UAP patients compared with those from SAP patients. This suggests that ER stress activation was related to the clinical situation, and the autopsy specimens were only slightly affected by postmortem protein degradation.

Among the oxysterols, 7-KC is most frequently detected at high levels in atherosclerotic plaques and in the plasma of patients with a high cardiovascular risk. To the best of our knowledge, however, 7-KC has not previously been detected in human atherosclerotic coronary artery sections by immunohistochemistry. It has been reported that 7-KC induces the
production of reactive oxygen species, activation of the UPR, and induction of apoptotic death in cultured human SMCs. \(^{28}\) We demonstrated that the fibrous caps of thin-cap atheroma were immunohistochemically positive for 7-KC, a finding consistent with the increase of ER stress/UPR markers.

Treatment of CASMCs with 7-KC induced ER stress and activation of the UPR, findings that were consistent with the results of a previous study on aortic SMCs, \(^{28}\) and these changes also occurred in THP-1 cells. This 7-KC–induced cellular damage was prevented by antioxidants (N-acetylcysteine and glutathione), which was also consistent with a previous report. \(^{36}\) Accordingly, the present findings suggest that an increase of ER stress due to 7-KC induces apoptosis of SMCs and macrophages through the production of reactive oxygen species.

ER stress induces apoptosis via the CHOP-, JNK–, and caspase-12–dependent signaling pathways. \(^{16}\) CHOP is mainly induced at the transcriptional level by ER stress, \(^{12,27}\) after which its overexpression leads to apoptosis. \(^{11,16,38}\) CHOP knockout mice show normal development and normal fertility but exhibit less apoptosis in response to ER stress. \(^{16,21}\) Thus, detection of the induction of CHOP indicates an increase of ER-initiated apoptosis. Although the direct transcriptional target of CHOP has not been found, \(^{39}\) the Bcl-2 pathway may be involved in the downstream connection between CHOP and apoptosis. \(^{28,39}\) Caspase-12 is only activated by ER stress. \(^{13,16,18}\) Although caspase-12 has been cloned in mice and rats, it is not yet possible to explore the role of this caspase in humans. \(^{40}\) JNK is 1 of the stress-activated protein kinases that has been shown to induce apoptosis in response to ER stress. \(^{13,16}\) We demonstrated that TUNEL-positive SMCs and macrophages were significantly increased in the fibrous cap, with CHOP (but not JNK) being induced simultaneously. Treatment of CASMCs or THP-1 cells with 7-KC induced CHOP, whereas knockdown of CHOP expression by siRNA led to a decrease of TUNEL-positive cells after exposure to 7-KC. Because CHOP is a transcription factor that specifically mediates ER-initiated apoptosis, the induction of CHOP in ruptured and unstable plaques supports the activation of ER-initiated apoptosis. However, our autopsy study could not exclude the possibility that the cells underwent apoptosis independently of CHOP, whereas the TUNEL assay gave false-positive results in the clinical specimens.

Unfortunately, we could not confirm whether or not the relationship between thinning of the fibrous cap and ER stress was causative because of the lack of a suitable animal model of plaque rupture. On the other hand, together with the present finding that 7-KC induced ER stress, the possibility that ER stress causes plaque vulnerability is also supported by the following reports. In cultured peritoneal macrophages, excessive accumulation of free cholesterol has been found to initiate ER stress, increase CHOP expression, and induce apoptosis. \(^{21}\) Moreover, in vivo studies with apoE–/– mice have shown that lesional necrosis can be diminished by a decrease in the cholesterol level. \(^{22}\) In addition, the present study demonstrated that expression of ER chaperones was upregulated to a similar extent in macrophages surrounding the necrotic cores of thick-cap atheroma, thin-cap atheroma, and ruptured plaques, which suggests that ER stress may contribute to the progression of plaque vulnerability by inducing macrophage apoptosis.

In conclusion, the present findings support the possibility that ER stress and/or the UPR induces apoptosis of SMCs and macrophages, thus increasing the vulnerability of coronary artery plaques, which may lead to ACS and a fatal outcome in patients with coronary artery disease.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Most of the acute clinical manifestations of coronary atherosclerosis result from plaque rupture that produces the acute coronary syndrome, and apoptosis is considered to be essential for plaque rupture. The endoplasmic reticulum (ER) is 1 of the largest cellular organelles and has multiple functions, such as regulating the folding of proteins. Various stimuli cause ER stress, including ischemia, heat shock, mutation, increased protein synthesis, and reactive oxygen species, all of which can potentially lead to ER dysfunction. The ER responds to stresses by upregulation of ER chaperones, but prolonged ER stress eventually causes apoptosis. However, the influence of ER stress and apoptosis on rupture of unstable coronary plaques remains unclear. We examined histological sections from coronary artery segments obtained at autopsy from 71 patients and atherectomy specimens obtained from 40 patients. Smooth muscle cells and macrophages in the fibrous caps of thin-cap atheroma and ruptured plaques showed a marked increase of ER chaperone expression and apoptotic cells. ER chaperones also showed higher expression in atherectomy specimens from patients with unstable angina pectoris than from those with stable angina. We also investigated possible signaling pathways for ER-initiated apoptosis and found that the C/EBP homologous protein (a transcription factor induced by ER stress)–dependent pathway was activated in unstable plaques. In addition, knockdown of C/EBP homologous protein expression by small interfering RNA decreased ER stress-dependent death of cultured coronary artery smooth muscle cells and THP-1 cells. Increased ER stress occurs in unstable plaques. Our findings suggest that ER stress-induced apoptosis may contribute to plaque vulnerability.

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