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The Role of Endoplasmic Reticulum Stress in the Progression of Atherosclerosis

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This Review is part of a thematic series on **Endoplasmic Reticulum Stress and Cardiac Diseases**, which includes the following articles:

What Is the Role of ER Stress in the Heart? Introduction and Series Overview [*Circ Res.* 2010;107:15–18]

The Role of Endoplasmic Reticulum Stress in the Progression of Atherosclerosis

Therapeutic Targets of ER Stress in Cardiovascular Disease

ER Stress and Cardiac Hypertrophy/Heart Failure

Biology of ER Stress in the Heart

Masafumi Kitakaze, Guest Editor

The Role of Endoplasmic Reticulum Stress in the Progression of Atherosclerosis

Ira Tabas

Abstract: Prolonged activation of the endoplasmic reticulum (ER) stress pathway known as the unfolded protein response (UPR) can lead to cell pathology and subsequent tissue dysfunction. There is now ample evidence that the UPR is chronically activated in atherosclerotic lesional cells, particularly advanced lesional macrophages and endothelial cells. The stressors in advanced lesions that can lead to prolonged activation of the UPR include oxidative stress, oxysterols, and high levels of intracellular cholesterol and saturated fatty acids. Importantly, these arterial wall stressors may be especially prominent in the settings of obesity, insulin resistance, and diabetes, all of which promote the clinical progression of atherosclerosis. In the case of macrophages, prolonged ER stress triggers apoptosis, which in turn leads to plaque necrosis if the apoptotic cells are not rapidly cleared. ER stress–induced endothelial cell apoptosis may also contribute to plaque progression. Another potentially important proatherogenic effect of prolonged ER stress is activation of inflammatory pathways in macrophages and, perhaps in response to atheroprone shear stress, endothelial cells. Although exciting work over the last decade has begun to shed light on the mechanisms and in vivo relevance of ER stress–driven atherosclerosis, much more work is needed to fully understand this area and to enable an informed approach to therapeutic translation. (*Circ Res.* 2010;107:839-850.)

Key Words: ER stress ■ unfolded protein response ■ atherosclerosis ■ macrophage ■ endothelial cells ■ apoptosis ■ CHOP ■ XBP1

Atherosclerotic vascular disease is the leading cause of death in the industrialized world, soon to be worldwide.^{1,2} Atherogenesis begins with the subendothelial retention of apolipoprotein (apo)B-containing lipoproteins in focal areas of the arterial subendothelium.^{3,4} These lipoproteins, perhaps after oxidative or other types of modification, then trigger a series of maladaptive inflammatory responses.^{3–7} Key among these responses is the attraction of blood-borne monocytes to activated endothelial cells (ECs) overlying areas of lipoprotein retention, followed by monocyte differentiation into

macrophages. The macrophages internalize the retained lipoproteins, leading to foam cell formation, and the macrophages become activated and inflammatory.^{6–9} Dendritic cells, T cells, mast cells, B cells, and possibly neutrophils also enter the growing lesion and contribute to the inflammatory response to retained lipoproteins. As lesions develop, the inflammatory response amplifies and does not resolve, which is likely a result of the continuing and even accelerated process of lipoprotein retention.^{3,10,11} As such, the subendothelial space expands with cells and extracellular matrix.

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Non-standard Abbreviations and Acronyms	
aP2	adipocyte fatty acid-binding protein-2 (macrophage fatty acid-binding protein-4)
apoB	apolipoprotein B
ApoE	apolipoprotein E
AMPK	AMP-activated protein kinase
ATF	activating transcription factor
CaMK	calcium/calmodulin-dependent protein kinase
CHOP	CEBP-homologous protein
EC	endothelial cell
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERO	endoplasmic reticulum oxidase
IP3R	inositol 1,4,5-triphosphate-activated receptor
IRE	inositol requiring
JNK	c-Jun amino-terminal kinase
LDL	low-density lipoprotein
Ldlr	low-density lipoprotein receptor
Lp(a)	lipoprotein(a)
NF-κB	nuclear factor κ B
oxPL	oxidized phospholipid
PBA	4-phenyl butyric acid
PERK	RNA-dependent protein kinase-like endoplasmic reticulum kinase
PRR	pattern-recognition receptor
ROS	reactive oxygen species
SERCA	sarco-/endoplasmic reticulum calcium-dependent ATPase
SFA	saturated fatty acid
SMC	smooth muscle cell
TLR	toll-like receptor
TUDCA	tauroursodeoxycholic acid
UPR	unfolded protein response
VSMC	vascular smooth muscle cell
XBP1	<i>Xho</i> I site-binding protein 1

In the majority of lesions, several processes prevent the key clinical consequence of atherosclerosis, namely, acute thrombotic vascular occlusion.¹² Among these are outward remodeling of the vessel wall, which maintains the patency of the arterial lumen; phagocytic clearance (“efferocytosis”) of dead cells, mostly apoptotic macrophages, which prevents plaque necrosis; and scar formation by collagen-producing intimal smooth muscle cells (myofibroblasts), which helps defend against matrix protease-mediated erosion or rupture of the intima into the lumen.^{13–16} However, in a small percentage of lesions, one or more of these processes fail, leading sequentially to plaque erosion or rupture, exposure of the blood to coagulation and thrombotic factors in necrotic lesions, and acute luminal thrombosis.^{13,17,18} Examples of cellular processes that lead to this failure are increased lesional macrophage apoptosis, defective efferocytosis, and death of collagen-producing intimal smooth muscle cells.^{13–16}

As is obvious from the above summary, a complex interplay among cell biological and physiological factors pro-

motes the initiation and progression of atherosclerosis. At one level, non-arterial wall systemic factors, especially those leading to elevated levels of circulating apoB lipoproteins, play essential roles. At the level of the arterial wall, exogenous and endogenous factors contribute to atherogenesis and plaque progression by promoting (1) apoB lipoprotein retention and modification; (2) activation of ECs; (3) entry and activation of inflammatory cells, notably macrophages; (4) entry and proliferation of intimal smooth muscle cells; (5) regulation of collagen biosynthesis and collagen turnover; (6) alterations in intimal cell death and in clearance of the dead cells; and (7) activation of coagulation factors and platelets. As will become evident in this review, a number of these processes can be influenced by prolonged endoplasmic reticulum (ER) stress.

Physiological and Pathophysiologic Endoplasmic Reticulum Stress

Over the last decade, ER stress has emerged as a factor that is relevant to a number of systemic and arterial wall factors that promote atherosclerosis. As reviewed in this series, ER stress represents a response by cells to transient or prolonged perturbations in ER function, especially function related to protein synthesis, calcium regulation, and intracellular redox potential.^{19–21} ER stress signaling, often referred to as the unfolded protein response (UPR), is triggered by 3 upstream proteins, IRE1 (inositol requiring 1), activating transcription factor (ATF)6, and PERK (RNA-dependent protein kinase-like endoplasmic reticulum kinase). IRE1, by promoting the expression of XBP1, and ATF6 play key roles in chaperone production, which helps relieve physiological and pathophysiologic imbalances between nascent proteins and the chaperones required to ensure proper protein folding and assembly. The XBP1 pathway also promotes the degradation of misfolded proteins. Other IRE1 pathways can lead to apoptosis (below) and mRNA degradation.²² PERK, by phosphorylating eIF2 α (eukaryotic initiation factor 2 α), temporally slows protein translation, which allows perturbations in protein translation to be corrected in an optimal manner. PERK, through phospho-eIF2 α -mediated translational upregulation of ATF4, also leads to the induction of CEBP-homologous protein (CHOP) (also known as GADD153 [growth arrest and DNA damage 153]; gene name *Ddit3*), which participates in various corrective functions during transient ER stress. However, the fact that CHOP-deficient mice develop normally and exhibit good health²³ suggests that many of the salutary functions of CHOP may be redundant with other ER stress effectors in the laboratory animal setting. As described below, prolonged expression of CHOP is a potent inducer of apoptosis.

Despite the essential beneficial functions of the UPR during transient ER stress, pathologically chronic ER stress often leads to tissue dysfunction and disease.¹⁹ ER stress can be prolonged by chronic disturbances in protein folding, oxidative stress, and other processes that lead to sustained ER dysfunction. In one scenario relevant to atherosclerosis, chronic ER stress affects systemic risk factors at the level of hepatic lipid metabolism and pancreatic β -cell function, particularly in the settings of obesity, insulin resistance, and

diabetes. This topic is not discussed here, but a number of excellent reviews in this area have been published.^{24–28} Rather, this review focuses on the effects of ER stress on plaque cells, particularly macrophages and ECs. Relevant to this topic is the general principle that 2 segments of the UPR can trigger pathological cell death. Chronic activation of IRE1 promotes apoptosis through the signal transducers ASK1 (apoptosis signal-regulating kinase 1) and JNK (c-Jun amino-terminal kinase) and by altering the balance and activity of Bcl family members.²⁹ Prolonged elevation of CHOP triggers apoptosis through effects on intracellular calcium metabolism and by alterations in Bcl family members.^{23,29–32} There is increasing *in vitro* and *in vivo* evidence that ER stress–induced apoptosis of intimal cells, notably macrophages, plays an important role in atherosclerotic plaque progression. In addition, chronic ER stress can adversely affect EC biology. This review summarizes the latest findings related to these topics and discusses therapeutic implications and future directions.

Proatherogenic Effects of ER Stress in Endothelial and Smooth Muscle Cells

Endothelial Cells

As reviewed above, alterations in endothelial function and protein expression play important roles in attracting inflammatory cells during atherogenesis. Lusis and colleagues³³ treated cultured human aortic ECs with the UPR activator tunicamycin and found induction of interleukin-8, interleukin-6, MCP-1 (monocyte chemoattractant protein 1), and CXCL3 (chemokine CXC motif ligand 3). The expression of these molecules could be blocked by gene silencing of ATF4 and/or XBP1 (*Xho*I site-binding protein 1). UPR activation in ECs was also induced by oxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-*sn*-3-glycero-phosphorylcholine), an oxidized phospholipid (oxPL) found in advanced atherosclerotic lesions. A possible *in vivo* link was suggested by the finding that oxPL-rich areas of human lesional endothelium showed evidence of UPR activation.³³ Interestingly, the ability of oxPAPC to activate the UPR in human aortic ECs differed significantly among individual donors, and this phenotypic variation was associated with genetic variation in a locus affecting action of USP16, a histone H2A deubiquitinase.³⁴

Atherosclerosis occurs at sites of disturbed blood flow, and biomechanical transduction of disturbed flow through the ECs at these sites may be a key process in promoting proatherogenic EC alterations in response to retained lipoproteins.³⁵ In this context, the Davies group found that the ER stress transducers IRE1 α , ATF6 α , and XBP1 were increased in ECs from atherosusceptible regions in normal swine aorta.³⁶ In cultured ECs, atheroprone flow (shear stress) increased the expression of the UPR effector GRP78 (78-kDa glucose-regulated protein) via a pathway involving the mitogen-activated protein kinase p38 and the integrin α 2 β 1.³⁷ There is also some evidence that endothelial ER stress in prelesional areas is exacerbated in the setting of diabetes, perhaps because of hyperglycemia-driven accumulation of glucosamine in the cells.³⁸ Xu and colleagues³⁹ also found that endothelial XBP1 expression was increased by disturbed

flow in cultured ECs. This finding was correlated with endothelial proliferation, which may reflect a protective response. However, when XBP1 was overexpressed in ECs, a key endothelial junctional adhesion protein, VE-cadherin, was decreased and endothelial apoptosis ensued. In *ApoE*^{-/-} mice, endothelial XBP1 expression correlated with areas of lesion severity, and when XBP1 was overexpressed in lesions, atherosclerosis was accelerated. One interpretation of these findings is that transient, limited XBP1 induction protects ECs against disturbed flow, whereas chronically elevated levels of XBP1 damages the cells and thereby promotes atherosclerosis. However, it is also possible that other UPR effectors that are induced in atheroprone endothelium may play a proatherogenic role. For example, there is evidence that prelesional ECs in these sites are primed through changes in gene expression for subsequent lipopolysaccharide- or high-fat diet–induced activation of the nuclear factor (NF)- κ B pathway.^{40,41} Given the links between the UPR and NF- κ B activation,⁴² it is possible that the early ER stress response may be involved in these changes. Further mechanistic investigation and, most importantly, endothelial-specific XBP1-targeting studies in mouse models of atherosclerosis will be needed to support this idea.

Other possible atherorelevant inducers of pathological ER stress in ECs include homocysteine and modified forms of low-density lipoprotein (LDL). Homocysteine induces ER stress–induced apoptosis in cultured human umbilical vein ECs. Whereas one group showed that disabling mutants of IRE1 can block apoptosis in this model,⁴³ another group showed that overexpression of a PERK pathway effector called TDAG51 (T-cell death–associated gene 51), which is upregulated in homocysteine-treated ECs, can trigger a form of detachment-mediated cell death.^{44,45} Hyperhomocysteinemia is associated with atherothrombotic vascular disease in humans and accelerated atherosclerosis and heightened ER stress in mouse models of atherosclerosis.^{46,47} When hyperhomocysteinemic, fat-fed rabbits were treated with taurine to reduce homocysteine levels in the plasma, coronary artery atherosclerosis, endothelial apoptosis, and endothelial CHOP were decreased.⁴⁸ However, the proatherogenic effect of homocysteine in animal models likely involves other lesional cell types in addition to ECs,⁴⁷ and how much of the proatherogenic effect of hyperhomocysteinemia is attributable to prolonged ER stress awaits genetic causation studies *in vivo*.

Various forms of LDL modification, such as through oxidation, glycation, or lipid hydrolysis, may occur during the course of atherogenesis.⁷ Oxidized and glycated LDL is an inducer of the UPR in cultured ECs through a mechanism involving disturbed ER calcium metabolism.⁴⁹ In particular, the oxidized-glycated LDL induces oxidative stress in ECs, which in turn inhibits the ER calcium pump, sarcoplasmic/ER calcium-dependent ATPase (SERCA). Another link between SERCA oxidation and ER stress was revealed by a mouse model lacking an endogenous inhibitor of SERCA oxidation called AMP-activated protein kinase (AMPK) α 2. The authors showed that *Ldlr*^{-/-} mice lacking this protein had increased lesional ER stress and atherosclerosis.⁵⁰ Finally, LDL hydrolyzed by secretory phospholipase A₂ induces the UPR in cultured ECs, which in turn stimulates inflammatory cytokine production through activation of p38.⁵¹

Smooth Muscle Cells

Less is known about the potential atherogenic role of ER stress in vascular smooth muscle cells (VSMCs) than in ECs or macrophages. In theory, ER stress–induced smooth muscle cell (SMC) apoptosis could lead to decreased collagen production and thus thinning of the protective collagen cap in advanced lesions.⁵² A proof-of-concept study using an ER stress–inducing drug called bortezomib was used to test the hypothesis that UPR-induced apoptosis in VSMCs could trigger death of the cells and thereby adversely affect collagen production in lesions. In this study, bortezomib, which is a proteasome inhibitor, was shown to cause ER stress and apoptosis in cultured VSMCs but not in macrophages.⁵³ In aortic explants from *Apoe*^{-/-} mice, bortezomib induced the UPR, including CHOP, and cell death was observed in regions that were populated with SMCs. In fat-fed *Apoe*^{-/-} mice, bortezomib was associated with a marked decrease in VSMCs, a modest decrease in collagen, and a marked increase in necrotic cores.⁵³ Whether or not these effects were actually caused by UPR induction remains to be shown.

Possible endogenous inducers of ER stress–induced apoptosis in VSMCs include 7-ketocholesterol, unesterified cholesterol, homocysteine, and glucosamine. Exposure of human coronary artery–derived VSMCs to 7-ketocholesterol, which is found in rupture-prone shoulder regions of thin-capped human coronary atheromata, led to the induction of CHOP and UPR chaperones.⁵⁴ Induction of these effectors was associated with the accumulation of reactive oxygen species (ROS) and could be blocked by the antioxidant *N*-acetylcysteine. Most importantly, 7-ketocholesterol induced apoptosis in the VSMCs, and silencing of CHOP through small interfering RNA blocked apoptosis.⁵⁴ Unesterified cholesterol induces ER stress–induced apoptosis in cultured SMCs,⁵⁵ as it does in macrophages,⁵⁶ but there is little evidence that SMCs in atherosclerotic lesions accumulate large amounts of unesterified cholesterol. Evidence that the UPR inducer homocysteine could affect SMCs comes from the study described in the previous section examining hyperhomocysteinemic *Apoe*^{-/-} mice.⁴⁷ The authors found evidence of UPR induction in intimal SMCs in these mice, which may be mediated through changes in ER calcium release.⁵⁷ Moreover, homocysteine activates SREBP-2 (sterol response element binding protein-2) in cultured VSMCs, leading to an increase in intracellular lipid accumulation.^{58,59} Finally, intracellular glucosamine, which is elevated in the setting of diabetes, has been shown to induce the UPR effector Grp78 (78-kDa glucose-regulated protein) in cultured human aortic SMCs, but whether this is a protective or maladaptive response was not addressed.⁶⁰ Future studies are needed to determine more precisely the extent to which the UPR is activated in lesional SMCs during lesion development and how ER-stressed SMCs might affect atherosclerosis progression.

Proatherogenic Effects of ER Stress in Macrophages

Evidence and Roles for ER Stress in Advanced Lesional Macrophages

Initial work by Feng et al³⁰ and then Austin and colleagues⁶¹ demonstrated that macrophages are prominent among athero-

sclerotic lesional cells undergoing ER stress in general, and expressing CHOP in particular, during the progression of atherosclerosis in chow-fed or Western diet–fed *Apoe*^{-/-} mice. These data provided evidence that CHOP expression increases as lesions progress, a concept that was later supported and expanded to human atherosclerosis in an important study by Myoishi et al.⁶² These investigators examined human coronary artery lesions in autopsy samples and fresh human carotid endarterectomy specimens for lesion stage, UPR markers, and apoptosis, as measured by TUNEL assay. In both sets of human lesions, the authors found a striking relationship among advanced lesion stage, CHOP expression, and lesional apoptosis such that only advanced, “vulnerable” plaques showed evidence of robust CHOP expression and apoptosis.

The human data support a working model developed by the Tabas laboratory and others over the last decade to explain how ER stress–induced macrophage apoptosis is a key event in the generation of necrotic cores and thus clinically dangerous plaques.^{11,15,63} In early lesions, macrophage apoptosis is difficult to detect, because the apoptotic cells are rapidly cleared by neighboring macrophages through a phagocytic process called “efferocytosis.”^{15,63} Efferocytosis engulfs and safely destroys apoptotic cells before they become necrotic, and the process also triggers a distinct antiinflammatory response involving cytokines such as interleukin-10 and transforming growth factor- β .⁶⁴ Indeed, genetic manipulations that promote macrophage apoptosis in early lesions actually suppress lesion cellularity and inhibit plaque progression through this efferocytic mechanism, and vice versa.^{65,66} In advanced lesions, however, the situation is very different. First, apoptosis is almost certainly increased and is triggered by mechanisms unique to the milieu of the advanced plaque, notably chronic ER stress.⁶⁷ Second, efferocytosis is not as efficient as in normal physiology, and so the apoptotic cells become secondarily necrotic, and the aforementioned antiinflammatory response does not occur.⁶⁸ The cumulative result is the formation of inflammatory necrotic cores and, potentially, plaque disruption and acute luminal thrombosis.^{11,15,63}

Experimental Models of ER Stress in Cultured Macrophages

Mechanistic data with cultured macrophages and molecular genetic causation data in vivo using mouse models of atherosclerosis have revealed a critical role for the UPR in advanced lesional macrophage death and plaque necrosis.^{30,69–71} The cell culture studies have used experimental proof-of-concept inducers of ER stress, as well as stressors that are likely to be relevant to advanced lesions. Examples of the former type include the protein glycosylation inhibitor tunicamycin⁴² and the SERCA inhibitor thapsigargin.⁷² The atherorelevant inducers are chosen from among the molecules and processes in advanced lesions that can lead to prolonged UPR activation, such as high levels of intracellular unesterified cholesterol, oxysterols, oxidant stress, hypoxia, and peroxynitrite.⁷³ One type of cultured macrophage model uses robust ER stress, which can be achieved with thapsigargin or

7-ketocholesterol, an ER stressor that is the most abundant oxysterol in human atherosclerotic lesions.³¹

Another type of model takes into account the possibility of more subtle ER stress in vivo. In this model, macrophage apoptosis is induced by the combination of a low-dose ER stressor and an atherorelevant “second hit,” each of which are unable to induce apoptosis by themselves. Examples include low-dose 7-ketocholesterol, thapsigargin, or the peroxynitrite donor SIN-1 as the ER stressor and combinatorial pattern-recognition receptor (PRR) activation as the second hit.^{74–76} Combinatorial PRR activation triggers apoptosis in the setting of subtle ER stress by both enhancing apoptotic pathways, including a proapoptotic NADPH oxidase–ROS pathway, and by suppressing cell survival pathways that are compensatorily triggered by ER stress (see below).^{72,75,76} This pathway of macrophage apoptosis may have evolved as a host defense mechanism against disease-causing organisms that require living macrophages to survive.⁷⁷ One example of combinatorial PRR activation that can trigger apoptosis in macrophages undergoing subtle ER stress are activators of the type A scavenger receptor (SRA) and toll-like receptor (TLR)4, such as oxidized LDL or acetylated LDL.⁷⁵ A second example are activators of CD36 and TLR2/6 heterodimer, such as oxPLs, oxidized LDL, or saturated fatty acids (SFAs).^{70,76} Another variation of this model is a “combined, two-hit” scenario in which macrophages unable to reesterify lipoprotein-derived cholesterol are incubated with atherogenic lipoproteins. In this setting, the lipoproteins activate TLRs and scavenger receptors at the cell surface, and lipoprotein-derived unesterified cholesterol that accumulates in the ER membrane activates the UPR.^{74,75,78} For each of these models, there is evidence in vivo. For example, advanced lesional macrophage apoptosis and plaque necrosis are blocked in advanced murine atheromata that have genetically induced deletion or inhibition of scavenger receptors,⁷⁹ TLRs,⁷⁶ or cholesterol trafficking to the ER membrane.⁸⁰

Mechanisms of CHOP-Induced Apoptosis in ER-Stressed Macrophages

Using one or more of these cultured macrophage models, mechanistic studies have shown that a full apoptosis response requires the CHOP pathway upstream and activation of both the Fas and mitochondrial pathways of apoptosis downstream.^{30,81,82} Recent studies have provided evidence for specific molecular signaling pathways linking CHOP with the downstream apoptosis processes (Figure 1). In particular, CHOP promotes apoptosis primarily by stimulating a persistent release of calcium from ER stores.^{31,32} The mechanism involves activation of inositol 1,4,5-triphosphate-activated receptor (IP3R)-mediated calcium release by the action of the CHOP transcriptional target ER oxidase (ERO)1 α .³¹ Evidence suggests that ERO1 α activates IP3R1 by hyperoxidizing the ER lumen, leading to disulfide bond formation in a luminal loop of IP3R1, which in turn increases IP3R1 calcium channel activity.^{83,84} The persistent increase in cytoplasmic calcium activates calcium/calmodulin-dependent protein kinase (CaMK)II, which then activates a number of proapoptotic pathways, including those involving the death receptor Fas, the proapoptotic pathway mediated by release of

apoptogenic factors from the mitochondria, a proapoptotic pathway involving signal transducer and activator of transcription-1 (STAT1), and NADPH oxidase–mediated ROS^{32,76} (G. Li, C. Scull, L. Ozcan, and I.T., manuscript submitted for publication). Most importantly, interruption of these events, most notably through gene targeting of CHOP, lessens advanced lesional macrophages apoptosis and plaque necrosis in both the *Ldlr*^{-/-} and *Apoe*^{-/-} models of atherosclerosis.^{69,71} In vitro studies have suggested another role of prolonged CHOP induction in macrophages, namely, activation of inflammatory signaling pathways.⁸⁵ In particular, prolonged CHOP expression leads to induction of interleukin-6 through an extracellular signal-regulated kinase (ERK)1/2 pathway.⁸⁵ Interestingly, CHOP-induced inflammation in macrophages can be stimulated by anti-HIV protease inhibitors, which are associated with accelerated atherothrombotic vascular disease.⁸⁶

CHOP deletion does not fully suppress apoptosis in ER-stressed macrophages, and so other mechanisms of ER stress–induced apoptosis involving the IRE1–JNK pathway and/or alterations in Bcl family members may also be involved (see the introduction). In this regard, Li et al⁸⁷ showed that IRE1 α was activated (phosphorylated) in lesional extracts from Western diet–fed *Apoe*^{-/-} mice and that cholesterol-induced apoptosis was partially suppressed in cultured macrophages treated with *Irela* small interfering RNA or with a JNK inhibitor. However, even here, there may be a link with the CHOP pathway, because silencing of IRE1 α in ER-stressed macrophages was associated with a decrease in CHOP expression.⁸⁷ In terms of Bcl family members, Bax levels are increased in the cholesterol model of ER stress–induced macrophage apoptosis.⁸² Moreover, deletion of Bcl2 in macrophages renders them more susceptible to ER stress–induced apoptosis in vitro and is associated with accelerated advanced lesional apoptosis and plaque necrosis in Western diet–fed *Apoe*^{-/-} mice.⁸⁸ Defining CHOP-independent pathways of ER stress–induced macrophage apoptosis in advanced atherosclerosis is an important goal for the future.

Another important consideration in the study of ER stress–induced macrophage apoptosis is the fact that the initial ER stress response is often accompanied by activation of cell survival signaling pathways, which then become deactivated soon before apoptosis occurs. Examples of cell survival modules that are activated and then eventually fail in macrophages exposed to prolonged ER stress include pathways mediated by interferon- β , Akt, NF- κ B, p38 α , ERK,^{72,75,89–92} and autophagy⁹³ (X. Liao, J. Sluimer, B. Levine, and I.T., unpublished data 2010). Thus, understanding how these pathways are activated and, more importantly, why they eventually fail represents a critical area in this field.

Insulin Resistance, Saturated Fatty Acids, and Lipoprotein(a) As Inducers of ER Stress–Induced Macrophage Apoptosis

The concept that ER stress–/CHOP-induced macrophage apoptosis is a critical step in necrotic plaque formation may have particular relevance to the rapidly growing epidemic of atherothrombotic vascular disease driven by insulin resistance and diabetes.^{92,94} Indeed, atherosclerotic lesions in

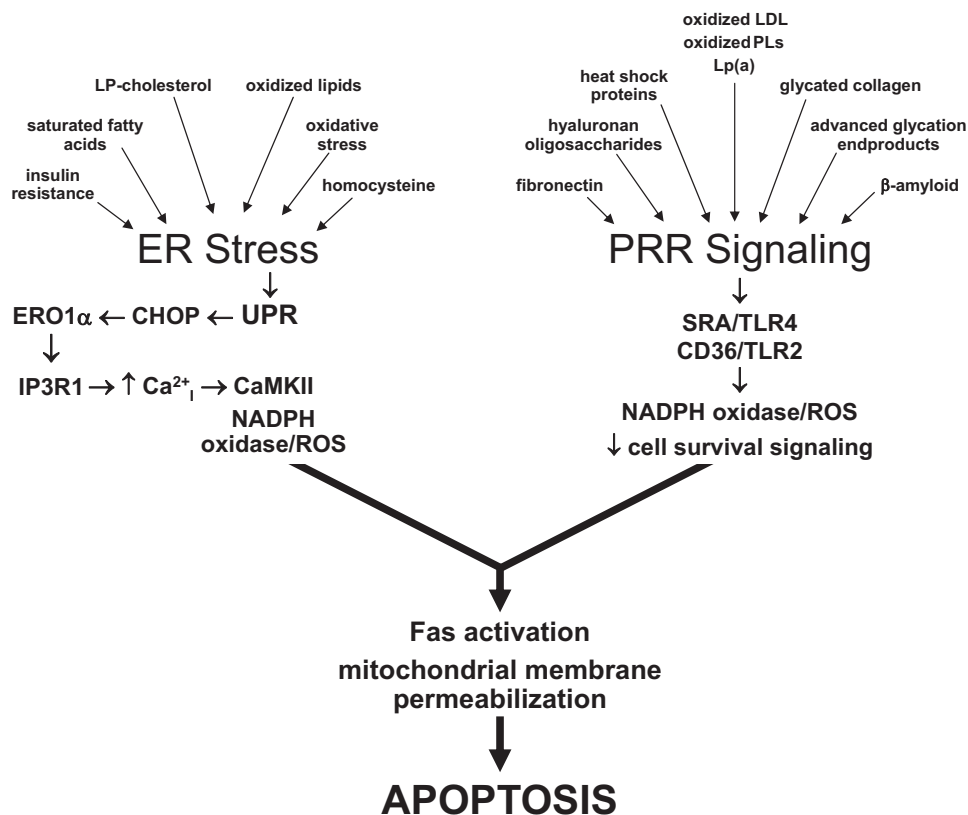


Figure 1. Model of ER stress-induced apoptosis in advanced lesional macrophages. Advanced lesions contain a number of molecules and processes that are known ER stressors. Activation of the UPR in macrophages leads to elevated levels of CHOP, which, through a pathway involving the CHOP target ERO1 α and IP3R-mediated calcium release, leads to elevation in cytosolic calcium (Ca^{2+}) and activation of CaMKII. CaMKII triggers apoptosis execution pathways through a number of mechanisms, including NADPH oxidase activation and subsequent ROS generation. In vivo evidence suggests that this scenario is necessary but not sufficient by itself for apoptosis. Rather, apoptosis in ER-stressed macrophages may require a “second hit” consisting of combinatorial PRR activation, and there are many factors in advanced lesions that can activate PRRs. The mechanism by which PRR activation tips the balance toward apoptosis in ER-stressed macrophages involves amplification of apoptosis pathways, such as NADPH oxidase-mediated ROS generation and suppression of ER stress-induced compensatory cell survival pathways. See text for details.

diabetic subjects are characterized by especially large necrotic cores, even when corrected for overall lesion size.⁹² In this context, recent studies have shown that a potent inducer of prolonged ER stress in macrophages is insulin resistance.⁹⁵ Macrophages have a functional insulin receptor signaling pathway, and downregulation of insulin receptor signaling caused by hyperinsulinemia promotes ER stress and apoptosis in these cells.⁹⁶ The mechanism involves processes that elevate cytosolic calcium through SERCA inhibition (see above).⁹⁵ Moreover, apoptosis is further exacerbated in the setting of macrophage insulin resistance through a mechanism involving suppression of the NF- κ B cell survival pathway.⁹⁰ Most importantly, both advanced lesional macrophage apoptosis and plaque necrosis are increased in a model of macrophage insulin resistance in Western diet-fed *Ldlr*^{-/-} mice.⁹⁶

Obesity and insulin resistance may further promote ER stress in lesional macrophages through elevations of SFAs.^{70,76} Although the mechanism of SFA-induced ER stress is not fully known, SFAs lower the fluidity of the ER membrane bilayer, which is a known inducer of the UPR.^{78,97} Recent work has suggested that an intracellular “lipid chaperone” called macrophage fatty acid-binding protein-4, also known

as adipocyte fatty acid-binding protein aP2, mediates SFA-induced ER stress and apoptosis in macrophages.⁷⁰ Previous work had shown that *Apoe*^{-/-} mice deficient for aP2 are protected against atherosclerosis.⁹⁸ In the new study, macrophages lacking aP2 were shown to be protected from palmitate-induced ER stress and apoptosis, and this protection was associated with activation of the transcription factor liver X receptor (LXR) α . LXR α is an inducer of an enzyme that converts SFAs into monounsaturated fatty acids, which are much less potent inducers of ER stress. Thus, aP2, by preventing SFA desaturation, is a mediator of SFA-induced ER stress in macrophages. In the Western diet-fed *Apoe*^{-/-} model, aP2 deficiency resulted in decreased P-PERK, XBP1, and apoptosis in macrophage-rich regions of atherosclerotic lesions. Whether aP2 deficiency protects against plaque necrosis, an important consequence of ER stress-induced macrophage apoptosis, has not yet been demonstrated. Furthermore, molecular genetic proof that the protective effects of aP2 deficiency in Western diet-fed *Apoe*^{-/-} mice are mediated by suppression of SFA-induced UPR activation remains to be demonstrated, although the role of the UPR was supported by data from a drug experiment in this study, as is discussed below.

The ability of oxPLs to trigger apoptosis in ER-stressed macrophages (above) may provide a clue to a 60-year-old mystery in heart disease. Approximately 25% to 30% of whites have elevated levels of a lipoprotein called lipoprotein(a) [Lp(a)], which is an LDL-like lipoprotein with a covalently bound kringle-containing protein called apolipoprotein(a).⁹⁹ Based on many clinical studies, including large epidemiological and human genetic studies, Lp(a) has been shown to be a potent, independent risk factor for advanced atherothrombotic vascular disease.^{100–103} Nonetheless, the mechanisms linking Lp(a) with specific cellular events associated with advanced plaque progression and necrosis, the sine qua non of clinically relevant atherosclerotic disease, are not known. Important observations over the last decade have revealed that Lp(a) is a major carrier of oxPLs in human plasma,¹⁰⁴ which, based on the two-hit ER stress–PRR model described above, raised the question of whether Lp(a) could trigger apoptosis in ER-stressed macrophages. Exciting new data have shown that Lp(a), but not native LDL, is a potent inducer of apoptosis in ER-stressed macrophages, but not unstressed macrophages.⁷⁶ As with oxPLs, Lp(a)-induced apoptosis depended on TLR2/6, CD36, and oxidative stress. In vivo support for the role of Lp(a) in advanced lesional macrophage death is provided by a transgenic rabbit model in which modest plasma levels of Lp(a) markedly promoted plaque necrosis without affecting en face lesion area.¹⁰⁵ Additional mechanistic and in vivo studies, including studies with human lesional material, will be needed to provide further support for this new concept.

Therapeutic Implications

Given the role of prolonged ER stress in a number of important diseases, notably those associated with advanced age and obesity, there has been increasing interest in therapeutic strategies focused on relieving ER stress, including for the purpose of preventing atherosclerosis.^{25,106,107} In this regard, compounds that seem to act as so-called “chemical chaperones” may be useful in decreasing the adverse effects of prolonged ER stress.^{106,107} Among these are 2 compounds (4-phenyl butyric acid [PBA] and tauroursodeoxycholic acid [TUDCA]) that have been tested in animal studies for the aforementioned purpose and used in humans for a variety of disorders.¹⁰⁸

As mentioned in the introduction, prolonged ER stress in the setting of obesity can lead to systemic atherosclerotic risk factors related to insulin resistance, and both compounds have been shown to have benefit in relieving insulin resistance in experimental mouse models of obesity.¹⁰⁸ In terms of direct effects on the arterial wall, the mouse study described above on SFA/aP2-induced ER stress in lesional macrophages showed that PBA relieved palmitic acid-induced ER stress and apoptosis in cultured macrophages and decreased lesion area and both lesional ER stress and apoptosis in Western diet-fed *Apoe*^{-/-} mice.⁷⁰ In a different setting, namely, diabetes-induced atherosclerosis in male hamsters, PBA failed to relieve ER stress or atherosclerosis.¹⁰⁹ TUDCA treatment was used in yet another model of ER stress in atherosclerosis (ie, Western diet-fed *Ampka2*^{-/-}*Ldlr*^{-/-} mice [above]) and was found to have beneficial effects on

both lesional ER stress and atherosclerosis lesion area.⁵¹ However, definitive proof that the antiatherogenic mechanisms of PBA and TUDCA are linked to relief of ER stress per se is lacking.

Statins are the mainstay of preventative therapy for atherothrombotic vascular disease in humans.¹¹⁰ Although their predominant and perhaps sole beneficial effect is through their ability to lower apoB lipoproteins in the circulation, thus decreasing arterial wall lipoprotein retention, the possibility of so-called “pleiotropic” protective effects by mechanisms other than through lowering plasma cholesterol is a topic of great interest in the field.¹¹⁰ One study showed a possible beneficial effect of statins in preventing SFA-induced ER stress in a human monocyte–macrophage cell line and in decreasing in ER stress in lesions of statin-treated *Ldlr*^{-/-} mice.¹¹¹ However, much more mechanistic and in vivo work is needed to further substantiate this point. Furthermore, any effect of these drugs on lesional ER stress may be through the decrease in exposure of lesional cells, predominantly macrophages, to excess apoB lipoprotein–derived cholesterol.⁵⁶

A proposed strategy of preventative treatment that has not yet shown to be beneficial in humans (ie, antioxidant therapy) is also relevant to a discussion of therapeutically targeting ER stress in lesions, because oxidative stress can be both a cause and a consequence of prolonged ER stress.¹¹² For example, the aforementioned example of ER stress being triggered by oxidation of the ER calcium pump SERCA can be relieved by the antioxidants apocynin and tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl or 4-hydroxy-tempo),⁵⁰ and tempol has been shown to decrease lesional ER stress and atherosclerosis in Western diet-fed *Apoe*^{-/-} and *Apmka2*^{-/-} *Apoe*^{-/-} mice (above).⁴⁹ Moreover, oxPLs, Lp(a), SFAs, and 7-ketocholesterol activate NADPH oxidase and induce oxidative stress in cultured macrophages⁷⁶ (G. Li, C. Scull, L. Ozcan, and I.T., manuscript submitted for publication, 2010). These processes are crucial for ER stress–induced apoptosis in macrophages and can be prevented by inhibitors of NADPH oxidase and by the antioxidant *N*-acetylcysteine⁷⁶ (G. Li, C. Scull, L. Ozcan, and I.T., manuscript submitted for publication, 2010). The major antioxidant that has been tested in humans is vitamin E, with most studies showing no beneficial effect.¹¹³ However, vitamin E may not target the specific mechanisms of oxidative stress associated with ER stress–induced perturbations in lesional cells.¹¹⁴ For example, the Tabas laboratory found that whereas *N*-acetylcysteine was effective in preventing ER stress–induced macrophage apoptosis, vitamin E was not (G. Li and I.T., unpublished data). Another strategy may be to target specific oxidative enzymes that may play critical roles in oxidative stress–induced cell death. In addition to NADPH oxidase, the ER oxidase ERO1 α plays a crucial role in CHOP-mediated death in ER-stressed macrophages,³¹ and Ron and colleagues¹¹⁵ have recently described small molecular inhibitors of this enzyme that protected mouse embryonic fibroblasts from ER stress–induced cell death.

A discussion of “anti-ER stress therapy” to prevent or treat atherosclerosis must consider several important points. First, as mentioned above, the beneficial effects of PBA and TUDCA, and particularly antioxidants, in mouse models of

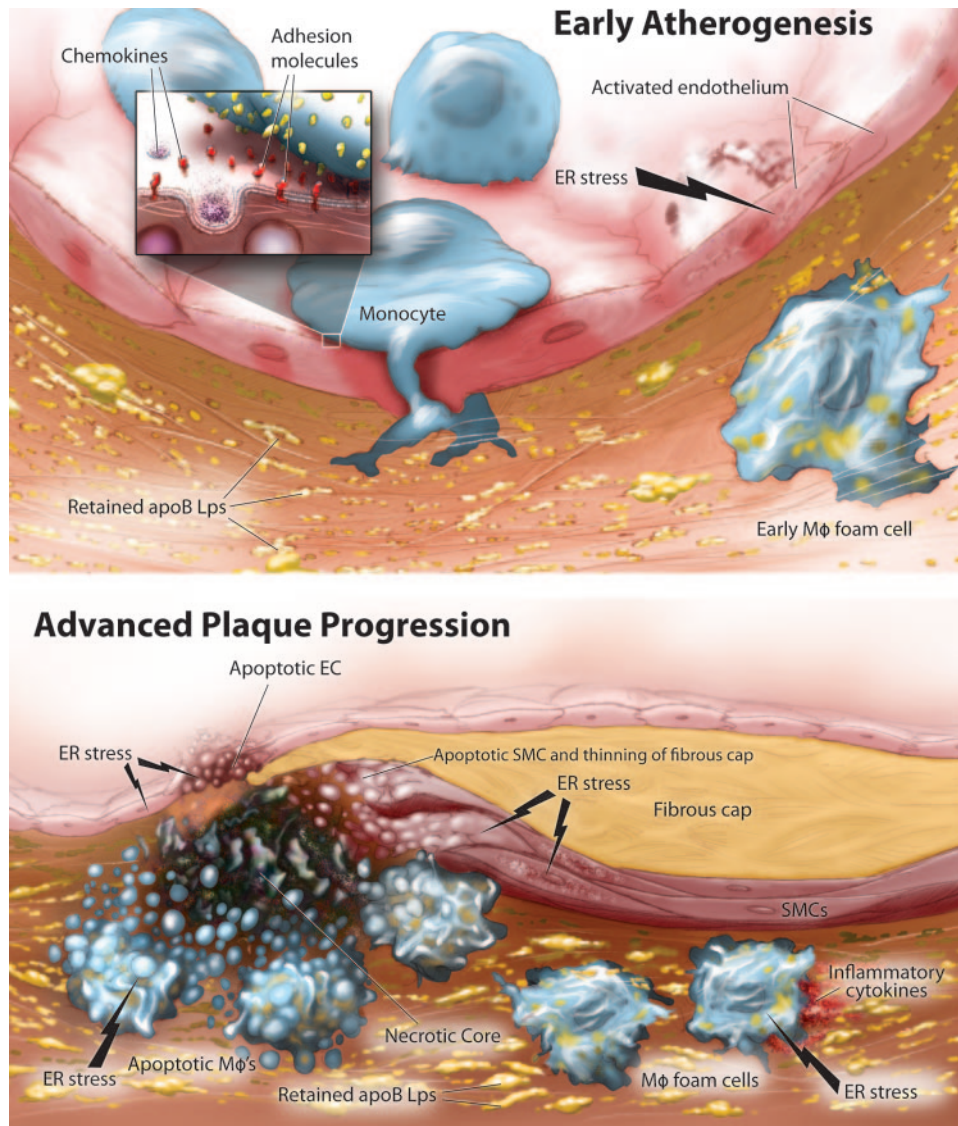


Figure 2. Possible roles of prolonged ER stress in early atherogenesis and advanced plaque progression. **A**, In early atherogenesis, extracellular matrix–retained and modified apoB-containing lipoproteins (LPs) trigger the expression of adhesion molecules and chemokines in ECs (“EC activation”), leading to attraction of monocytes and other inflammatory cells to the nascent lesion. Early lesional ECs, perhaps in response to disturbances in laminar blood flow at atherosusceptible sites, show evidence of ER stress, which may further promote EC activation. **B**, In advanced lesions, ER stress is prominent in macrophages (Mφs) and can lead to inflammation and apoptosis in these cells. When apoptotic macrophages are not rapidly cleared by neighboring phagocytes, they become secondarily necrotic and lead to the generation of the necrotic core, a key feature of clinically dangerous plaques. ER stress in advanced lesions may also cause the death of ECs, which may further amplify plaque progression and disruption, and cell death of smooth muscle cells, which may contribute to the thinning of the protective fibrous cap. See text for details. (Illustration credit: Cameron Slayden, Cosmocyte, Savage, Md.)

atherosclerosis have not yet been proven to be related to their ER stress–relieving properties. For example, PBA has a number of other biological actions, including histone deacetylase inhibitor activity.¹¹⁶ Second, the UPR is an essential protective pathway in cells, and attempts to relieve ER stress by blocking this pathway will likely cause adverse effects unless the approach is targeted and limited. Moreover, different cell types in lesions may have different mechanisms of ER stress–induced damage. For example, prolonged CHOP expression appears to be an important cause of macrophage apoptosis,^{30,62,69,71} whereas excessive induction of XBP1 has been shown to induce apoptosis in ECs.³⁹ However, physiological levels of XBP1 may play a protective role in ECs

subjected to atherogenic perturbations in blood flow.³⁶ Thus, the success of an ER stress–relieving strategy will likely depend on which branch of the UPR is targeted and whether it can prevent excessive UPR activation without perturbing physiological ER stress signaling. In this light, recent developments in understanding how IRE1 α functions to promote cell death versus cell survival at a protein structural level raise the possibility of much more specific drugs that can block IRE1 α –dependent cell death while retaining the protective effects of the IRE1 α –XBP1–chaperone pathway.^{22,115} Third, the process in atherosclerosis that actually leads to clinical disease is the development of necrotic, thin-capped plaques.^{13,17,18} Clinical studies in humans have shown that this type of

“vulnerable” plaque morphology is much more predictive of atherothrombotic vascular disease than overall plaque size.¹¹⁷ However, most animal studies use decrease in lesion area rather than improvements in plaque morphology as the measure of drug effectiveness. An ER stress–induced process specifically associated with plaque necrosis is advanced lesional macrophage apoptosis.¹¹ However, even here, the situation is complex in terms of therapy, because although preventing death of advanced lesional macrophages may decrease plaque necrosis, it would also increase the number of living macrophages, which may promote plaque disruption through other mechanisms, such as secretion of matrix-degrading proteases.¹¹⁸ In summary, careful consideration to mechanism, site of action, potency, and adverse effects of ER stress–relieving therapy will be essential if this strategy is ever to be useful in preventing atherothrombotic vascular disease.

Summary and Future Directions

Prolonged ER stress has been identified as a pathogenic mechanism in a large number of disease processes, particularly chronic diseases associated with aging, obesity, and diabetes. Atherothrombotic vascular disease is particularly important to consider in this arena, because ER stress can adversely affect both systemic atherosclerotic risk factors and cell biological processes occurring at the level of the arterial wall (Figure 2). With regard to the arterial wall, the focus of this review, there is now increasing *in vitro* and *in vivo* evidence that prolonged ER stress is an important cause of macrophage and possibly EC apoptosis in advanced lesions (Figure 2B). Additional ER stress–mediated proinflammatory effects in these cells may also affect early atherogenesis (Figure 2A). Nonetheless, much more work is needed to truly understand the role of ER stress in atherosclerosis and to enable a well-informed effort at translating this knowledge into useful therapeutic strategies. Given the complexity of the multiple branches and functional consequences of ER stress signaling, a major goal over the next several years will be to define which branches of the UPR are activated and to elucidate the consequences of individual or integrated branch activation. Moreover, each cell type in atherosclerotic lesion development plays distinct roles, and these roles vary throughout the course of atherogenesis. Thus, determining the effects of interrupting prolonged ER stress in individual cell types at various stages during lesion development is a critical goal for the future. The development and use of conditionally gene-targeted mice will be essential to meet this goal. Temporal control over UPR gene silencing will also help avoid the potential confounding effects of compensatory alterations attributable to germline deletion. In terms of linking ER stress with clinically relevant atherosclerosis progression, mouse models of atherosclerosis are useful to study atherogenesis from lesion initiation up to the stage of necrotic plaques, but they are not a good model for plaque disruption or acute luminal thrombosis.¹¹⁹ Although future mouse models may help address this problem, there is no substitute for human studies. Continuing work on describing what happens to ER stress pathways in different cell types in human lesions, particularly the types of lesions that are likely

to cause clinical disease, will help inform animal studies. In addition, more precise causation information may be gleaned from genome-wide association studies¹²⁰ and UPR gene resequencing studies in which subjects with accelerated atherothrombotic vascular disease are compared with age-matched control subjects. Only through the knowledge gained from this combined approach will we be able to fully understand the roles of ER stress in atherosclerosis, to determine whether specific elements of prolonged ER stress are rationale drug targets, and to intelligently approach drug discovery and testing in this area.

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