

Association of Multiple Cellular Stress Pathways With Accelerated Atherosclerosis in Hyperhomocysteinemic Apolipoprotein E-Deficient Mice

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Background—A causal relation between hyperhomocysteinemia (HHcy) and accelerated atherosclerosis has been established in apolipoprotein E-deficient (apoE^{-/-}) mice. Although several cellular stress mechanisms have been proposed to explain the atherogenic effects of HHcy, including oxidative stress, endoplasmic reticulum (ER) stress, and inflammation, their association with atherogenesis has not been completely elucidated.

Methods and Results—ApoE^{-/-} mice were fed a control or a high-methionine (HM) diet for 4 (early lesion group) or 18 (advanced lesion group) weeks to induce HHcy. Total plasma homocysteine levels and atherosclerotic lesion size were significantly increased in early and advanced lesion groups fed the HM diet compared with control groups. Markers of ER stress (GRP78/94, phospho-PERK), oxidative stress (HSP70), and inflammation (phospho-IκB-α) were assessed by immunohistochemical staining of these atherosclerotic lesions. GRP78/94, HSP70, and phospho-IκB-α immunostaining were significantly increased in the advanced lesion group fed the HM diet compared with the control group. HSP47, an ER-resident molecular chaperone involved in collagen folding and secretion, was also increased in advanced lesions of mice fed the HM diet. GRP78/94 and HSP47 were predominantly localized to the smooth muscle cell-rich fibrous cap, whereas HSP70 and phospho-IκB-α were observed in the lipid-rich necrotic core. Increased HSP70 and phospho-IκB-α immunostaining in advanced lesions of mice fed the HM diet are consistent with enhanced carotid artery dihydroethidium staining. Interestingly, GRP78/94 and phospho-PERK were markedly increased in macrophage foam cells from early lesions of mice fed the control or the HM diet.

Conclusions—Multiple cellular stress pathways, including ER stress, are associated with atherosclerotic lesion development in apoE^{-/-} mice. (*Circulation*. 2004;110:207-213.)

Key Words: cells ■ atherosclerosis ■ lesion ■ apolipoproteins

Hyperhomocysteinemia (HHcy) is associated with increased risk for cardiovascular disease.^{1,2} Mild to moderate HHcy accelerates atherosclerotic lesion development in apolipoprotein E-deficient (apoE^{-/-}) mice with diet-induced HHcy,³⁻⁶ and supplementation of diets with B vitamins essential for homocysteine metabolism attenuates HHcy-induced atherosclerosis.^{4,5} Despite these studies supporting an atherogenic effect of HHcy, the cellular stress mechanisms by which HHcy accelerates atherosclerosis are not completely understood.

Several hypotheses have been proposed to explain the atherogenic effect of HHcy. First, the thiol group of homocysteine is readily oxidized in plasma to form reactive oxygen species (ROS), suggesting a mechanism involving oxidative

damage.⁷ However, this hypothesis does not explain why cysteine, a thiol-containing amino acid that is present in blood in a 20- to 25-fold-higher concentration than homocysteine and can also be readily oxidized to form ROS, is not considered a major risk factor for cardiovascular disease.⁸ The recent findings that heme oxygenase-1 (HO-1) and glutathione peroxidase (GPx) expression and activity are impaired in cultured vascular endothelial cells treated with homocysteine⁹⁻¹¹ suggest that HHcy inhibits the antioxidant potential of cells. This is particularly relevant to atherogenesis, given that (1) HHcy increases vascular dysfunction in GPx-deficient mice,¹² (2) overexpression of GPx attenuates homocysteine-induced endothelial dysfunction,¹³ and (3) overexpression of HO-1 inhibits the development of athero-

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sclerosis in apoE^{-/-} mice.¹⁴ Second, increased intracellular levels of homocysteine cause endoplasmic reticulum (ER) stress, leading to activation of the unfolded protein response (UPR).^{10,15} Although the UPR provides an adaptive response for survival against ER stress, severe or prolonged UPR activation can lead to apoptotic cell death. Furthermore, homocysteine-induced ER stress activates the sterol regulatory element-binding proteins, leading to lipid dysregulation and hepatic steatosis in mice with diet-induced HHcy.^{16,17} Third, atherosclerosis is considered a progressive inflammatory disease,¹⁸ and activation of nuclear factor kappa-B (NF- κ B), a proinflammatory factor, has been demonstrated in atherosclerotic lesions from patients with cardiovascular disease¹⁹ and apoE^{-/-} mice with diet-induced HHcy.⁵

To better define the cellular stress mechanisms by which HHcy accelerates atherogenesis, immunohistochemical markers of ER stress, oxidative stress, and inflammation were assessed in atherosclerotic lesions from the aortic root of apoE^{-/-} mice fed a control diet or a high-methionine (HM) diet to induce HHcy. Our results demonstrate that multiple cellular stress pathways, including ER stress, are associated with atherogenesis in apoE^{-/-} mice.

Methods

Materials

DL-Homocysteine, L-methionine, Mayer's hematoxylin and eosin, and oil red O were obtained from Sigma-Aldrich. GRP78/94 (anti-KDEL), HSP47, or HSP70 antibodies were obtained from StressGen Biotechnologies. GRP78 antibodies were obtained from Santa Cruz. Phospho-PERK or phospho-I κ B- α antibodies were obtained from Cell Signaling. Mac-3 antibodies were obtained from PharMingen. Antibodies directed against smooth muscle α -actin, β -actin, or proliferating cell nuclear antigen (PCNA) were obtained from Sigma-Aldrich. Avidin biotin complex (ABC) systems for immunohistochemical staining and Nova red peroxidase substrate were obtained from Vector Laboratories.

Mice and Dietary Conditions

Female apoE^{-/-} mice, at 6 weeks of age, were fed a vitamin-defined diet (control, n=36) (Special Diet Services) or the same diet containing 1.35% (wt/wt) L-methionine (n=15) for 18 weeks (advanced lesion group). To investigate the early atherogenic effects of HHcy, mice were fed normal mouse chow (n=8) (Harlan Teklad) without or with 0.5% (wt/vol) L-methionine added to the drinking water (n=8) for 4 weeks (early lesion group). All procedures were approved by the National Animal Experiment Board (Denmark), McMaster University Animal Research Ethics Board, or the University of Iowa and Veterans Affairs Animal Care and Use Committees.

Plasma Homocysteine and Lipids

Plasma total homocysteine was quantified by means of gas chromatography-mass spectrometry (advanced lesion group)²⁰ or immunoassay (early lesion group).²¹ Plasma total cholesterol and triglycerides were assayed with the use of reagents from Roche Diagnostic or Sigma-Aldrich.

Tissue Sample Preparations, Staining, and Immunohistochemical Analysis

The mice were euthanized; hearts were flushed with 1 \times PBS and perfusion-fixed with 10% neutral buffered formalin.^{3,4} After removal, hearts (including the aortic root) were cut transversely and embedded in paraffin.²² Aortic root sections were collected on glass slides for the measurement of lesion size (orcein or hematoxylin and eosin staining) and immunohistochemical staining.

Immunohistochemical staining was performed with the use of the VECTASTAIN ABC System (Vector Laboratories). Sections stained with mouse primary antibodies (KDEL, HSP70, smooth muscle α -actin, phospho-I κ B- α , and PCNA) were performed with the use of the VECTOR MOM Immunodetection Kit to avoid cross-reactivity of mouse antibodies with mouse tissues. Sections were stained with diaminobenzidine or Nova red substrates and counterstained with hematoxylin for microscopic visualization. Unless otherwise stated, nonspecific immunostaining was not detected in sections stained with nonimmune IgG as the primary antibody or with the secondary antibody alone.

For immunofluorescence staining, sections were blocked with 5% normal donkey serum and incubated with primary antibodies, followed by Alexa 488-conjugated donkey anti-rabbit and Alexa 594-conjugated donkey anti-goat IgG (Molecular Probes).

Images were captured with the use of a CCD color video camera (Sony) and analyzed by means of Northern Exposure (Empix) and SigmaScan Pro. Semiquantitative assessment of immunostaining in atherosclerotic lesions was performed, as in our previous study.²³ Briefly, for intracellular immunopositive staining, such as that observed for HSP47 and PCNA, the percentage of positively stained cells was calculated. For extracellular immunostaining, such as that observed for phospho-I κ B- α and HSP70, the percentage of lesion area having positive immunostaining was calculated.

Dihydroethidium Fluorescence of Carotid Arteries

Carotid artery segments from apoE^{-/-} mice, fed the control (n=7) or the HHcy (n=7) diet, were stained with dihydroethidium.¹² Images were obtained with the use of a Bio-Rad MRC-1024 laser scanning confocal microscope and subsequently analyzed by means of NIH software (<http://rsb.info.nih.gov/nih-image>).

Statistical Analyses

Results were expressed as mean \pm SD. The unpaired Student *t* test was used to assess the difference between the experimental group and the control group. Probability values <0.05 were considered significant if not otherwise stated.

Results

Effect of Methionine Supplementation on Plasma Total Homocysteine and Lipids in ApoE^{-/-} Mice

Mean plasma total homocysteine levels were significantly elevated in apoE^{-/-} mice fed the HM diet for 4 weeks (early lesion group) (25.3 \pm 9.9 versus 6.1 \pm 1.3 μ mol/L, *P*<0.01) or 18 weeks (advanced lesion group) (53.6 \pm 8.7 versus 9.4 \pm 0.3 μ mol/L, *P*<0.01). Plasma cholesterol and triglyceride levels were similar in both control and HM groups except for a higher plasma cholesterol level (15.1 \pm 0.6 versus 13.8 \pm 0.3 mmol/L, *P*<0.05) in the advanced lesion group.

Effect of HHcy on Advanced Atherosclerotic Lesions in the Aortic Root of ApoE^{-/-} Mice

Mean atherosclerotic lesion size in the aortic root of the HM group was significantly larger (288.1 \pm 106.8 versus 175.1 \pm 62.6 μ m² \times 10³, *P*<0.01) (Figure 1, B versus A) compared with the control group. Increased oil red O staining (Figure 1, D versus C) and a significantly thicker fibrous cap containing more smooth muscle cells (*P*=0.026, Table and Figure 2, B versus A) were also observed in the HM group. In addition, the total number of cells positive for PCNA, a marker of cell proliferation, were markedly increased (*P*=0.007, Table and Figure 2, D versus C) in the atherosclerotic cap region from the HM group.

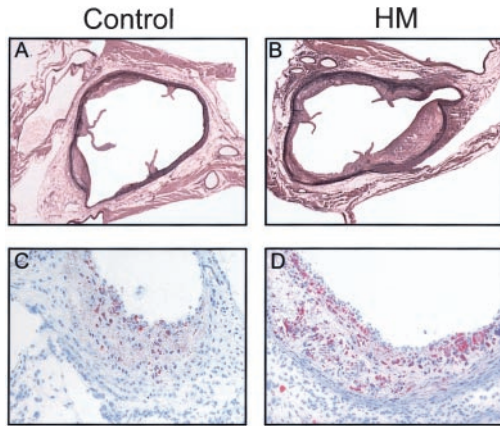


Figure 1. Morphology of advanced atherosclerotic lesions in aortic root of control or hyperhomocysteinemic apoE^{-/-} mice. Female apoE^{-/-} mice, 6 weeks of age, were fed control or HM diet for 18 weeks. Mice were euthanized, and sections were prepared at the aortic root and stained with orcein (A and B) or oil red O (C and D).

Expression of GRP78/94 and HSP47 in Advanced Atherosclerotic Lesions of ApoE^{-/-} Mice

Aortic root sections from the advanced lesion groups fed the control (Figure 3A) or the HM (Figure 3B) diet were immunostained with an anti-KDEL monoclonal antibody directed against GRP78/GRP94. Based on the density of staining, the majority of macrophage foam cells and smooth muscle cells stained positively for GRP78/94 in the atherosclerotic lesions and were predominantly situated in the fibrous cap. A significant increase ($P=0.002$, Table and Figure 3, B versus A) in GRP78/94 immunostaining within the advanced lesions was observed for the HM group.

HSP47, an ER-resident molecular chaperone involved in collagen processing and secretion,^{24,25} was expressed in smooth muscle cells situated within the fibrous cap (Figure 3, C and D). The total number of HSP47-positive smooth muscle cells was significantly higher ($P=0.0002$, Table and Figure 3, D versus C) in the HM group compared with control group.

Semiquantitative Assessment of Immunostaining in Early and Advanced Atherosclerotic Lesions From ApoE^{-/-} Mice Fed Control or HM Diet

Immunohistochemical staining	Control (Mean ± SD)	HM (Mean ± SD)	P
Advanced lesion group (18 wk)			
KDEL*	10.9 ± 4.3	15.9 ± 4.6	0.002
HSP47†	44.2 ± 22.2	72.0 ± 24.6	0.0002
HSP70*	2.6 ± 2.1	6.8 ± 2.4	0.035
P-IκB-α*	1.9 ± 0.3	3.8 ± 1.4	0.046
PCNA†	6.1 ± 3.1	18.6 ± 7.0	0.007
α-Actin*	14.8 ± 4.1	23.0 ± 3.8	0.026
Early lesion group (4 wk)			
KDEL*	21.6 ± 7.9	14.8 ± 9.0	0.08
Phosph-PERK*	10.3 ± 9.3	19.9 ± 7.1	0.056

*Percentage of lesion area positively stained.

†Percentage of positively stained cells per lesion.

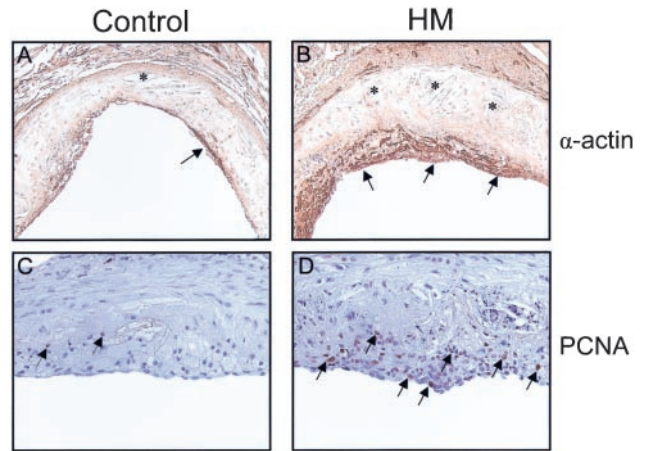


Figure 2. Identification of smooth muscle cells in atherosclerotic lesions from control or hyperhomocysteinemic apoE^{-/-} mice. Representative aortic root sections from apoE^{-/-} mice fed control or HM diet for 18 weeks were immunostained for smooth muscle α-actin (A and B) or PCNA (C and D). In A and B, smooth muscle cells are indicated by arrows; lipid-rich necrotic core is indicated by asterisks. In C and D, PCNA-positive smooth muscle cells are indicated by arrows.

Expression of HSP70 in Advanced Atherosclerotic Lesions of ApoE^{-/-} Mice

HSP70, an established cellular marker of oxidative stress, was observed in the necrotic lipid core of advanced lesions (Figure 4, A and B), a finding consistent with the localization of HSP70 in human and rabbit atherosclerotic lesions.^{26,27} HSP70 immunostaining was significantly higher ($P=0.035$, Table and Figure 4, B versus A) in the HM group compared with the control group. Consistent with the HSP70 immunostaining, dihydroethidium staining was elevated 2.7-fold ($P<0.05$ versus control group) in the carotid arteries of the HM group (Figure 4, C and D).

Expression of Phospho-IκB-α in Advanced Atherosclerotic Lesions of ApoE^{-/-} Mice

HHcy enhances the activation of NF-κB in the atherosclerotic lesions from apoE^{-/-} mice.⁵ To confirm and better define the precise localization of NF-κB activation, atherosclerotic le-

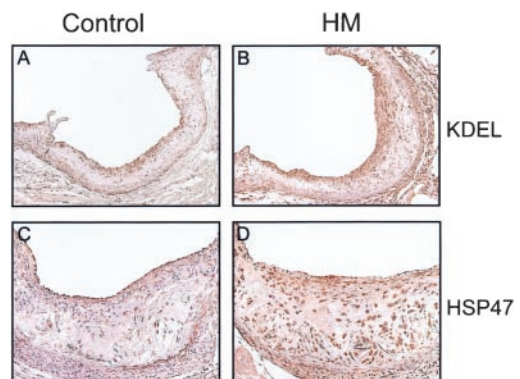


Figure 3. Expression of GRP78/94 (KDEL) and HSP47 in advanced atherosclerotic lesions from control or hyperhomocysteinemic apoE^{-/-} mice. Representative aortic root sections from apoE^{-/-} mice fed control or HM diet for 18 weeks were immunostained for GRP78/94 (A and B) or HSP47 (C and D).

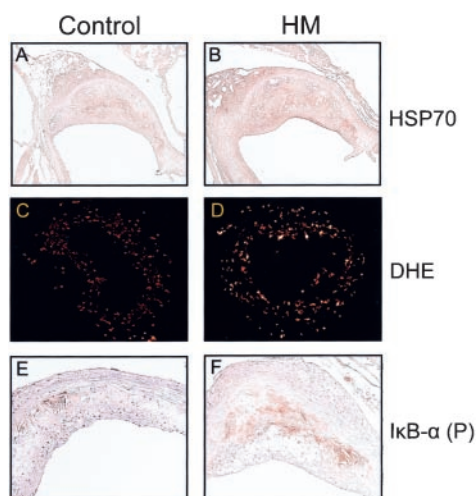


Figure 4. Evidence of oxidative stress and inflammation in atherosclerotic lesions or carotid arteries from control or hyperhomocysteinemic apoE^{-/-} mice. Representative aortic root sections from apoE^{-/-} mice fed control or HM diet for 18 weeks were immunostained for HSP70 (A and B) or phospho-IκB-α (E and F). In situ detection of superoxide is by dihydroethidium (DHE) fluorescence (C and D). Representative laser-scanning fluorescent confocal micrographs are of carotid artery of mice fed control (C) or HM (D) diet.

sions were immunostained with antibodies to phospho-IκB-α, a marker of NF-κB activation.²⁸ As shown in Figure 4, phospho-IκB-α was significantly increased ($P=0.046$, Table and Figure 4, F versus E) in advanced lesions of the HM group compared with the control group. Similar to that observed for HSP70, the majority of phospho-IκB-α immunostaining was confined to the necrotic lipid core.

Effect of HHcy on Early Atherosclerotic Lesion Development in ApoE^{-/-} Mice

Early atherosclerotic lesions, defined as fatty streaks, were observed in the aortic root from apoE^{-/-} mice fed the control or the HM diet for 4 weeks. Oil red O (Figure 5, A and B) and hematoxylin and eosin staining (Figure 5, C and D) as well as Mac 3 immunostaining (Figure 5, E and F) demonstrated that these early lesions consisted of macrophage foam cells. Mean lesion size was significantly increased (14.0 ± 7.5 versus $8.0 \pm 0.9 \mu\text{m}^2 \times 10^3$, $P < 0.05$) in the HM group compared with the control group. Unlike the weak immunostaining observed for HSP70 (Figure 6, A and B) or phospho-IκB-α (Figure 6, C and D), both GRP78/94 (Figure 6, E and F) and phospho-PERK (Figure 6, G and H) immunostaining were markedly increased in the early lesion-resident macrophage foam cells from mice fed the control or the HM diet. Although not significant, a trend toward increased phospho-PERK immunostaining in the early lesions was observed in the HM group (Table).

Colocalization studies indicated that the vast majority of macrophage foam cells in the early lesions of both control and HM groups stained for GRP78 and phospho-PERK (Figure 7). However, cells intensely stained for GRP78 showed weak immunostaining for phospho-PERK, and vice versa (Figure 7).

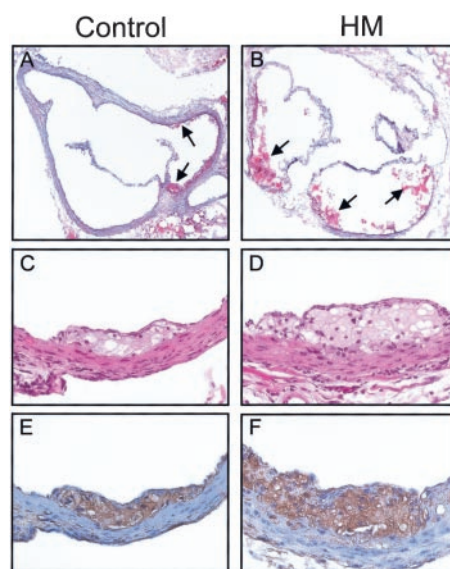


Figure 5. Early atherosclerotic lesion morphology in aortic root of control or hyperhomocysteinemic apoE^{-/-} mice. Female apoE^{-/-} mice, 6 weeks of age, were fed control or HM diet for 4 weeks. Mice were killed, and sections were prepared at the aortic root and stained with oil red O (A and B) or hematoxylin and eosin (C and D). Sections were also immunostained for macrophages with Mac-3 antibody (E and F).

Discussion

A causal relation between HHcy and accelerated atherosclerosis has been reported in apoE^{-/-} mice.³⁻⁶ Previous studies have observed evidence of increased oxidative stress and

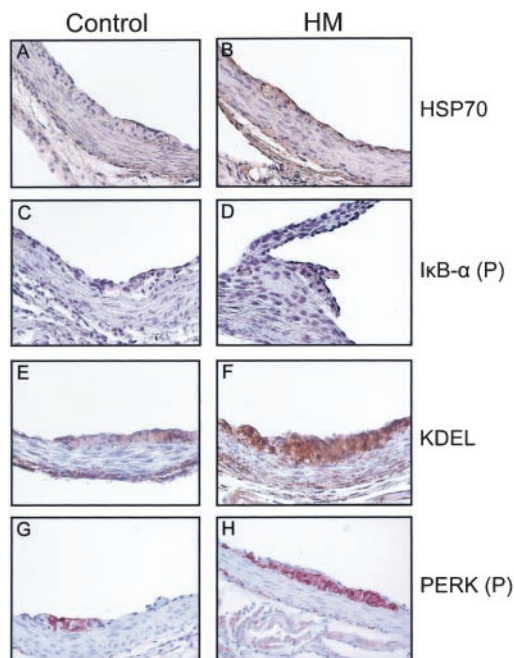


Figure 6. Expression of HSP70, phospho-IκB-α, GRP78/94 (KDEL), and phospho-PERK in early atherosclerotic lesions from control or hyperhomocysteinemic apoE^{-/-} mice. Representative aortic root sections from apoE^{-/-} mice fed control or HM diet for 4 weeks were immunostained for HSP70 (A and B), phospho-IκB-α (C and D), GRP78/94 (E and F), or phospho-PERK (G and H).

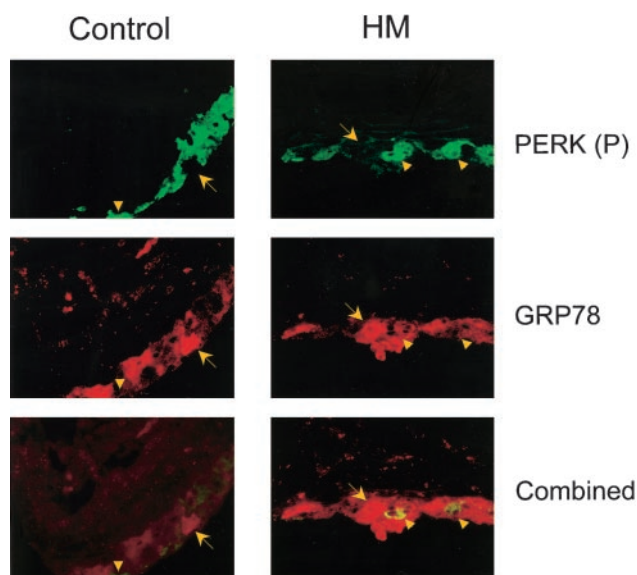


Figure 7. Colocalization of GRP78 and phospho-PERK in early atherosclerotic lesions from apoE^{-/-} mice. Representative aortic root sections from apoE^{-/-} mice fed control or HM diet for 4 weeks were immunostained for GRP78 (red) or phospho-PERK (green), and images were combined. Arrows indicate lesion-resident macrophages stained intensely for GRP78 but not phospho-PERK. Arrowheads indicate lesion-resident macrophages stained intensely for phospho-PERK but not GRP78.

inflammation in atherosclerotic lesions from apoE^{-/-} mice with diet-induced HHcy.⁵ We as well as others have also reported that homocysteine causes ER stress, activates the UPR, and increases the expression of UPR genes, including GRP78, GRP94, and GADD153.^{10,15} However, the association between ER stress and accelerated atherosclerosis was not examined. In this study, GRP78/94 immunostaining was significantly increased in advanced atherosclerotic lesions from apoE^{-/-} mice with diet-induced HHcy. Increased expression was observed primarily in macrophage foam cells and smooth muscle cells and correlated with a larger atherosclerotic lesion size.

HSP47 was also increased in smooth muscle cells from advanced atherosclerotic lesions of hyperhomocysteinemic apoE^{-/-} mice. Increased HSP47 expression has been observed in human atherosclerotic lesions,²⁹ and recent studies indicate that overexpression of HSP47 in smooth muscle cells enhances type I collagen processing and secretion.²⁴ Furthermore, our findings of increased HSP47 expression are consistent with several reports examining lesion development and HHcy. First, homocysteine enhances the synthesis and accumulation of smooth muscle cell collagen.³⁰ Second, HHcy is associated with an increase in the amount of collagen in lesions from apoE^{-/-} mice.³

The dramatic increase in GRP78/94 and phospho-PERK immunostaining in early lesion-resident macrophage foam cells from mice fed the control or the HM diet suggests that alterations in ER homeostasis are involved in the initial stages of atherogenesis and do not result from multiple confounding factors (ie, ischemia, cell death, depletion of nutrients), which would be expected in advanced lesions.

Furthermore, they suggest that other mechanisms independent of HHcy can promote ER stress. Recent studies have reported a significant increase in monocyte binding to the surface of aortic endothelium in rats with diet-induced HHcy.³¹ Given that B cell-to-plasma cell differentiation requires XBP-1,³² a transcriptional activator that amplifies the expression of UPR genes, including GRP78 and GRP94,³³ it is possible that the binding of monocytes to the endothelium and their subsequent differentiation into macrophage foam cells may require XBP-1, thereby accounting for the increased expression of GRP78/94. Because macrophage foam cells readily endocytose both native and oxidized forms of plasma LDL, the accumulation of lipids could potentially elicit an ER stress response. In support of this concept, Feng et al³⁴ showed that the ER is the site of cholesterol-induced cytotoxicity in cultured macrophages and that macrophage foam cells within advanced atherosclerotic lesions from apoE^{-/-} mice express GADD153, an established marker of ER stress.

The inverse correlation between GRP78 and phospho-PERK immunostaining in macrophage foam cells is similar to that observed for ischemic hippocampal C₁ neurons³⁵ and suggests temporal differences in UPR activation. These findings are also consistent with previous studies indicating that PERK phosphorylation is an early response to ER stress that is required for the inhibition of translation (through the phosphorylation of eIF-2 α) and transcriptional induction of UPR genes, including GRP78 and GRP94.³⁶

Oxidative stress is often cited as the mechanism responsible for the injurious effects of HHcy on cultured cells and intact tissues. HHcy activates NF- κ B in cultured endothelial cells, smooth muscle cells, and macrophages through oxidative stress,³⁷⁻³⁹ and induction of HHcy in apoE^{-/-} mice leads to vascular inflammation and NF- κ B activation.⁵ Thus, our observations of increased HSP70 and phospho-I κ B- α immunostaining in the advanced atherosclerotic lesions as well as increased dihydroethidium staining in the carotid arteries of hyperhomocysteinemic apoE^{-/-} mice are consistent with these findings. Given that homocysteine inhibits the activity and expression of several antioxidant enzymes, namely HO-1 and GpX,⁹⁻¹¹ HHcy could potentially mediate atherogenesis by sensitizing cells/tissues to oxidative stress.

Although considered as two independent cellular stress pathways, it is possible that ER stress and oxidative stress are related. Homocysteine is known to enhance intracellular production of superoxide, which is rapidly scavenged by nitric oxide to form peroxynitrite.^{8,11,40} Both superoxide and peroxynitrite contribute to the modification of tissues, resulting in the generation of lipid peroxides and in the case of peroxynitrite, the modification of proteins by tyrosine nitration and the formation of 3-nitrotyrosine. Given that peroxynitrite induces ER stress in cultured pancreatic and macrophage cell lines^{41,42} and that 3-nitrotyrosine staining precisely colocalizes with macrophages in atherosclerotic lesions from apoE^{-/-} mice,⁴³ the enhanced expression of ER stress markers observed in lesion-resident macrophage foam cells may result from impaired nitric oxide bioavailability. A second cellular process related to ER and oxidative stress is release of

calcium from intracellular stores, including the ER. Depletion of calcium from the ER is strongly associated with ER stress⁴⁴ and can potentially lead to the generation of ROS through the activation of cyclo-oxygenases and lipoxygenases.⁴⁵ Given that homocysteine elicits calcium release from cultured vascular smooth muscle cells,⁴⁶ it is conceivable that this could account for the increased expression of ER and oxidative stress markers in the atherosclerotic lesions from mice with HHcy. This mechanism is also consistent with the observation that activation of NF- κ B occurs in response to numerous cellular stresses, including ER stress.^{45,47} Clearly, other conditions involved in lesion formation, including shear stress, hypoxia, and the accumulation of lipid, could influence the activation of these cellular stress pathways.

Acknowledgments

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