Endoplasmic Reticulum Stress and Glycogen Synthase Kinase-3β Activation in Apolipoprotein E–Deficient Mouse Models of Accelerated Atherosclerosis

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Objective—The goal of this study was to examine the role of endoplasmic reticulum (ER) stress signaling and the contribution of glycogen synthase kinase (GSK)-3β activation in hyperglycemic, hyperhomocysteinemic, and high-fat–fed apolipoprotein E–deficient (apoE−/−) mouse models of accelerated atherosclerosis.

Methods and Results—Female apoE−/− mice received multiple low-dose injections of streptozotocin (40 μg/kg) to induce hyperglycemia, methionine-supplemented drinking water (0.5% wt/vol) to induce hyperhomocysteinemia, or a high-fat (21% milk fat+0.2% cholesterol) diet to induce relative dyslipidemia. A subset of mice from each group was supplemented with sodium valproate (625 mg/kg), a compound with GSK3 inhibitory activity. At 15 and 24 weeks of age, markers of ER stress, lipid accumulation, GSK3β phosphorylation, and GSK3β activity were analyzed in liver and aorta. Atherosclerotic lesions were examined and quantified. Hyperglycemia, hyperhomocysteinemia, and high-fat diet significantly enhanced GSK3β activity and also increased hepatic steatosis and atherosclerotic lesion volume compared with controls. Valproate supplementation blocked GSK3β activation and attenuated the development of atherosclerosis and the accumulation of hepatic lipids in each of the models examined. The mechanism by which GSK3β activity is regulated in these models likely involves alterations in phosphorylation at serine 9 and tyrosine 216.

Conclusion—These findings support the existence of a common mechanism of accelerated atherosclerosis involving ER stress signaling through activation of GSK3β. Furthermore, our results suggest that atherosclerosis can be attenuated by modulating GSK3β phosphorylation. (Arterioscler Thromb Vasc Biol. 2012;32:82-91.)

Key Words: atherosclerosis ■ diabetes mellitus ■ endoplasmic reticulum stress ■ glycogen synthase kinase 3 ■ apoE−/− mice

Atherosclerosis is a disease of the arterial wall that is characterized by inflammation and lipid accumulation. It is the underlying cause of cerebro- and cardiovascular disease that together account for approximately one third of the annual mortality in westernized societies. Risk factors for cardiovascular disease include diabetes mellitus, hypertension, dyslipidemia, hyperhomocysteinemia, abdominal obesity, smoking, and physical inactivity. However, the underlying cellular and molecular pathways that link specific risk factors to accelerated development of atherosclerosis remain unclear. Understanding the mechanisms that promote atherogenesis will be a major step toward the development of novel and effective antiatherosclerotic therapies.

Endoplasmic reticulum (ER) stress is defined as a condition in which the protein processing capacity of the ER is exceeded by nascent proteins, resulting in the accumulation of unfolded or misfolded proteins. The unfolded protein response (UPR) is a cellular self-defense mechanism that alleviates ER stress by limiting de novo protein synthesis, enhancing protein folding capacity, through the upregulation of specific ER chaperones, including glucose-related protein (GRP)78, GRP94, and calreticulin, and increasing the degradation of irreversibly misfolded proteins. If the UPR is insufficient to restore ER homeostasis, proapoptotic signaling factors, such as C/EBP homologous protein, can initiate programmed cell death.

Accumulating evidence supports a causative role for ER stress in the development or progression of atherosclerosis. First, several independent cardiovascular risk factors, including hyperglycemia, hyperhomocysteinemia, obesity, cigarette smoke, and elevated levels of unesterified cholesterol or palmitate, have been associated with activation of the UPR. Second, recent evidence shows that the UPR is specifically activated in atherosclerosis-susceptible regions of the arterial wall, before lesion development. Finally, ER stress–inducing agents can activate/dysregulate metabolic pathways that are directly involved in the development of atherosclerotic lesions. Specifically, we have shown that
conditions of ER stress promote lipid accumulation by activating the sterol regulatory element binding proteins, which are transcription factors that control lipid biosynthesis and uptake.\(^8\)\(^15\) ER stress–inducing agents also activate nuclear factor-κB, the transcription factor responsible for promoting inflammatory gene expression.\(^6\) In addition, ER stress can activate caspases and promote apoptosis of endothelial cells, macrophages, and other cell types.\(^12\) Together, lipid accumulation, inflammation, and endothelial/macrophage foam cell apoptosis are hallmark features of atherosclerosis.

The molecular pathways by which ER stress or the UPR activates proatherogenic responses are not known. One potential factor that is involved in ER stress signaling is glycogen synthase kinase (GSK)-3β, a kinase involved in many metabolic pathways.\(^18\) Conditions of ER stress have been shown to increase the activity of GSK3β in cultured cells. Furthermore, GSK3β has been shown to play a role in the activation of nuclear factor-κB both in vivo and in vitro.\(^19\)\(^20\) Recently, we have found that dietary supplementation with valproate, a small branch chain fatty acid with GSK3 inhibitory properties,\(^21\) can attenuate accelerated atherosclerosis in an streptozotocin-induced hyperglycemic apolipoprotein E–deficient (apoE \(^{-/-}\)) mouse model.\(^22\) These were the first reports to implicate GSK3 in the development of atherosclerosis. In this study, we investigated the potential role of ER stress and GSK3 in other models of accelerated atherosclerosis, including hyperhomocysteinemic and high-fat–fed mice. In addition, we examined the specific effects of atherosclerosis, including hyperhomocysteinemic and high-fat–fed mice. Finally, we examined the mechanisms by which these pro- and antiatherogenic stimuli regulate kinase activity in these different mouse models.

**Methods**

**Mouse Models**

Five-week-old female apoE \(^{-/-}\) (B6.129P2-ApoE\(^{m113c}\)) mice, purchased from the Jackson Laboratory (stock number 002050), were placed on a defined chow diet (TD92078, Harlan Teklad) and randomly divided into 4 groups (n=24/group). To induce hyperglycemia, 1 group was injected intraperitoneally with 10 injections of streptozotocin (40 mg/kg per day) as previously described.\(^23\) To induce hyperhomocysteinemia, L-methionine (0.5%, wt/vol) was added to drinking water. To induce relative dyslipidemia, mice were fed a high-fat diet containing 21% milk fat and 0.2% cholesterol (TD97363, Harlan Teklad). The final group remained on the standard chow diet. After 1 week, half of the mice in each group were switched to a diet supplemented with 625 mg/kg sodium valproate (standard plus valproate [TD02165] or high-fat plus valproate [TD06228]). All mice had unrestricted access to both food and water throughout the study. Mice were euthanized at 15 (hyperglycemic, hyperhomocysteinemic) or 24 (high-fat–fed) weeks of age, and tissues were collected for further analysis. The McMaster University Animal Research Ethics Board approved all procedures.

**Plasma Analysis**

Nonfasting whole blood glucose levels were measured using a DEX glucometer (Bayer). Plasma glucose and lipid levels were determined in nonfasted mice using the colorimetric diagnostic kits for total cholesterol and glucose purchased from Thermal DNA Inc. Plasma valproate concentrations were determined using the Axsym system (Abbott Laboratories). Plasma homocysteine levels were determined using the IMX system (Abbott Diagnostics). Plasma lipoproteins were separated by fast protein liquid chromatography, and total cholesterol was measured in each fraction.

**Immunohistochemical Analysis**

Mice were euthanized, and vasculature was flushed with 1× PBS and perfusion-fixed with 10% neutral buffered formalin. Liver and heart, including the aortic root, were removed and embedded in paraffin. Serial sections (4 μm) of aortic root were collected on precoated glass slides for measurement of lesion size (hematoxylin/eosin staining) or immunohistochemical staining.\(^24\) The Vectastain ABC System was used for immunohistochemical analysis. Sections were stained with primary antibodies and appropriate biotinylated secondary antibodies and visualized using Nova Red. Sections were counterstained with hematoxylin. Serial sections were stained with preimmune IgG, in place of primary antibodies, to control for nonspecific staining. Images were captured with an Olympus microscope and a 12.5-megapixel DP71 digital camera. Phospho-Tyr216-GSK3β antibody and total GSK3β antibody were purchased from BD Transductions (catalog Nos. 612312 and 610202, respectively), and phospho-Ser9-GSK3β antibody was purchased from Cell Signaling Technology (catalog No. 93235). Anti-KDEL antibody was purchased from Assay Designs (catalog No. SPA-827), and anti-C/EBP homologous protein antibody was purchased from Santa Cruz Biotechnology (catalog No. sc-575).

**Immunoblot Analysis**

Total protein lysates were solubilized in GSK3 buffer containing 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X, 10 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and PhosSTOP Phosphatase Inhibitor (Roche). Protein lysates were diluted in SDS-PAGE gel loading buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. After incubation with the appropriate primary and horseradish peroxidase–conjugated secondary antibodies (Life Technologies), membranes were developed using the Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore). Anti-KDEL (GRP78/94) antibody and protein disulphide-isomerase antibody were purchased from Assay Designs (catalog Nos. SPA-827 and SPA-891, respectively), and β-actin antibody was purchased from Sigma-Aldrich (catalog No. A3854). Phospho-Tyr216-GSK3β antibody and total GSK3β antibody were purchased from BD Transductions (catalog Nos. 612312 and 610202, respectively), and phospho-Ser9-GSK3β antibody was purchased from Cell Signaling Technology (catalog No. 93235).

**GSK3β Activity Assay**

GSK3β was immunoprecipitated from 900 μg of total mouse liver protein in GSK3 buffer using a monoclonal antibody specific for GSK3β (catalog No. 610202, BD Transductions) and Ultra Link immobilized Protein A Plus (Pierce). Kinase activity was measured by monitoring the incorporation of \(^{32}\)P onto phosphoglycogen synthase peptide-2 (Upstate Biotech). Briefly, immunoprecipitated GSK3β was combined with 15 μM p-GS-2 and 0.5 μCi/μL \([\gamma^32\text{P}]-\text{ATP}\) in a reaction mixture containing 20 mM MOPS, 50 μM EDTA, 0.25 mM Mg acetate, 5 mM MgCl\(_2\), 5 mM/L β-glycerol phosphate, 1 mM/L EGTA, 0.25 mM/L Na\(_2\)VO\(_4\), 0.25 mM/L dithiothreitol, and 35 μM/L ATP in a total volume of 40 μL. After 60 minutes at room temperature, samples were placed on ice and then spotted onto Whatman P81 phosphocellulose paper and washed 3 times with 0.75% o-phosphoric acid and once with acetone. \(^{32}\)P incorporation onto the substrate was determined by scintillation counting, and total counts minus background are reported.

**Lipid Staining**

Liver cryosections, 7 μm thick, were collected on slides and fixed in formal calcium for 30 minutes. Neutral lipids were visualized using Oil Red O as described previously.\(^6\) Relative amounts of lipid staining were quantified using ImageJ 1.35 software.
**Table. Metabolic Parameters in Apolipoprotein E\(^{-/-}\) Mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control, 15 wk</th>
<th>Control, +V</th>
<th>HG</th>
<th>HG + V</th>
<th>HH</th>
<th>HH + V</th>
<th>Control, 24 wk</th>
<th>HF</th>
<th>HF + V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>20.9±1.8</td>
<td>20.9±1.1</td>
<td>19.5±0.6</td>
<td>20.1±1.6</td>
<td>21.4±1.2</td>
<td>20.6±0.3</td>
<td>23.5±0.7</td>
<td>23.4±0.9</td>
<td>23.0±0.5</td>
</tr>
<tr>
<td>Lesion volume, (10^{-3}) mm(^3)</td>
<td>3.80±0.55</td>
<td>3.21±0.56</td>
<td>8.62±1.23(\dagger)</td>
<td>4.98±0.76(\dagger)</td>
<td>7.83±0.99(\dagger)</td>
<td>5.26±0.50(\dagger)</td>
<td>87.5±1.90</td>
<td>208±9.0(\dagger)</td>
<td>187±8.0 (\dagger)</td>
</tr>
</tbody>
</table>

**Cell Culture**

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum at 37°C and 5% CO\(_2\). Cells were treated with 8 \(\mu\)g/mL tunicamycin or 250 \(\mu\)mol/L bovine serum albumin–coupled palmitic acid for 8 hours. Oil Red O stain was applied to cells, and after washing with PBS and 60% ethanol, the cells were fixed in 2.5% paraformaldehyde. Images were captured with an Olympus microscope and a 12.5-megapixel DP71 digital camera. Intensity of staining was quantified using ImageJ software.

**Statistical Analysis**

Results are presented as the mean±standard deviation. The Student t test was used to assess differences between experimental groups and controls. Probability values of <0.05 were considered statistically significant.

**Results**

**Mouse Models of Accelerated Atherosclerosis**

We directly compared the effects of chronic hyperglycemia, hyperhomocysteinemia, or high-fat diet on ER stress levels, GSK3\(\beta\), and accelerated atherosclerosis in apoE\(^{-/-}\) mice (Table). Hyperglycemia was induced with multiple low-dose injections of streptozotocin (40 mg/kg body weight) in 5-week-old apoE\(^{-/-}\) mice. At 15 weeks of age, nonfasting blood glucose levels had increased to 23.2±5.6 mmol/L compared with 10.5±2.0 mmol/L in controls (\(P<0.05\)). Hyperhomocysteinemia was induced by adding 0.5% methionine (wt/vol) to the drinking water starting at 5 weeks of age. In the methionine-supplemented group, plasma homocysteine levels increased to 14.1±4.0 \(\mu\)mol/L compared with 4.6±0.8 \(\mu\)mol/L in controls (\(P<0.05\)). ApoE\(^{-/-}\) mice fed a high-fat diet containing 21% milk fat and 0.2% cholesterol had a significant increase in total plasma cholesterol (25.0±3.6 mmol/L versus 14.8±2.7 mmol/L in controls, \(P<0.05\)) and blood glucose (11.2±0.9 mmol/L versus 8.5±0.3 mmol/L in controls, \(P<0.05\)). Subsets of mice from each group were fed diets supplemented with 625 mg/kg sodium valproate. Valproate has been shown to inhibit GSK3\(\beta\) activity both in vitro and in vivo.\(^{21,22,25,26}\)

Plasma valproate concentration in supplemented mice was 33 to 54 \(\mu\)mol/L with the exception of the high-fat-diet mice in which it was significantly lower (7 \(\mu\)mol/L). The reason for this is not clear; however, it is possible that conditions of severe dyslipidemia may affect valproate uptake, clearance, or detection. Plasma homocysteine levels were lower in hyperglycemic mice compared with the normoglycemic treatment groups. This effect may result from insulin deficiency and has been previously noted in both animal models and young patients with diabetes mellitus.\(^{27,28}\) Valproate supplementation did not significantly affect blood glucose or plasma homocysteine concentrations in any of the groups (Table). In hyperglycemic and hyperhomocysteinemic mice, total plasma cholesterol and plasma lipid profiles were similar to those of control mice (Table and Supplemental Figure I, available online at http://atvb.ahajournals.org). As expected, a high-fat diet was associated with increased total plasma cholesterol relative to standard chow fed controls. Valproate supplementation did not affect plasma lipid profiles in hyperglycemic or hyperhomocysteinemic mice and was associated with a slight, nonsignificant decrease in very-low-density lipoprotein– and low-density lipoprotein–sized particles in high-fat–fed mice.

**The Effects of Valproate Supplementation on Accelerated Atherosclerosis**

Both hyperglycemia and hyperhomocysteinemia significantly increased mean atherosclerotic lesion volume at the aortic root of 15-week-old female apoE\(^{-/-}\) mice (Figure 1 and Table). Mice fed valproate-supplemented diets had significantly reduced cross-sectional lesion area and total mean lesion volume than the mice fed control diet (4.98±0.76 versus 8.62±1.23 \(\times 10^{-3}\) mm\(^3\), \(P<0.05\), in hyperglycemic
mice, and 5.26±0.50 versus 7.83±0.99×10⁻³ mm³, P<0.05, in hyperhomocysteinemic mice). Twenty-four-week-old mice fed a high-fat diet had significantly larger lesion volume compared with the mice fed the control diet (208±9 versus 87.5±1.9×10⁻³ mm³, P<0.05), and the littermates fed the high-fat diet supplemented with valproate had significantly reduced atherosclerotic lesion volume (187±8 versus 208±9×10⁻³ mm³, P<0.05) (Figure 1 and Table).

Atherosclerotic lesion areas were determined in descending aortas by staining en face with Sudan IV. Few lesions were found in the descending aortas of 15-week-old control, hyperglycemic, or hyperhomocysteinemic apoE⁻/⁻ mice (data not shown). At 24 weeks of age, however, there was detectable lesion development along the entire aorta. High-fat feeding increased atherosclerotic lesion areas approximately 3-fold relative to age-matched controls (Supplemental Figure II). Similar to the effects observed at the aortic sinus, valproate supplementation significantly reduced lesion area in the descending aorta (Supplemental Figure II).

**Hepatic and Aortic ER Stress Levels**

Immunohistochemical and immunoblot analyses were used to evaluate ER stress levels in the different experimental groups. Consistent with previous observations, hyperglycemia, hyperhomocysteinemia, and high-fat feeding were each associated with increased ER stress response protein expression. Immunoblot analysis of hepatic GRP78, GRP94, and protein disulphide-isomerase protein levels showed significantly elevated levels of all ER stress markers in each of the mouse models (Figure 2A and 2B). Aortic immunohistochemical analysis demonstrated increased expression of GRP78/94 and C/EBP homologous protein by probing with anti-KDEL and anti-C/EBP homologous protein antibodies, respectively (Figure 2C and 2D). Valproate supplementation did not significantly alter the intensity or staining pattern of the ER stress response proteins, nor did it significantly affect the

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**Figure 1.** The effect of valproate supplementation on accelerated atherosclerosis. A, Representative hematoxylin/eosin–stained cross-sections of the aortic sinus from 15-week-old control (C), hyperglycemic (HG), hyperhomocysteinemic (HH), or 24-week-old high-fat-diet–fed (HF) female apolipoprotein E-deficient mice. Arrows indicate atherosclerotic lesions. B, Quantification of atherosclerotic lesion area in the ascending aorta. V indicates valproate. C, Necrotic area within plaque as a percentage of total plaque size. *P<0.05 relative to control mice fed the nonsupplemented (control) diet. #:P<0.05 relative to the same group fed the control diet. n=9 to 10 per group (A and B), n=4 to 5 per group (C).
levels of ER stress markers in any of the models and tissues examined.

Lipid Accumulation and the Effects of Valproate Supplementation

Diabetes mellitus, severe hyperhomocysteinemia, and high-fat diet have each been associated with the accumulation of hepatic lipids, leading to nonalcoholic fatty liver.8,29 Conditions of ER stress have been shown to promote lipid accumulation in specific cell types, including hepatocytes, by dysregulating sterol regulatory element binding protein–controlled cholesterol and triglyceride biosynthesis and uptake.30 We quantified the relative hepatic neutral lipid levels in our hyperglycemic, hyperhomocysteinemic, and high-fat–fed apoE/H11002/H11002/H11002 mice by Oil Red O staining of frozen liver sections (Figure 3A). Results show a significant accumulation of hepatic lipid in each of the models, relative to controls. In hyperglycemic and hyperhomocysteinemic mice, valproate supplementation significantly reduced lipid concentrations to levels observed in the age matched control mice. High-fat–fed mice showed no significant change in hepatic lipid levels when supplemented with valproate (Figure 3B).

The Role of GSK3β in ER Stress–Induced Lipid Accumulation

To begin to examine the specific role of GSK3β in lipid accumulation, HepG2 cells were cultured in the presence of ER stress–inducing agents tunicamycin (8 g/mL) or palmitic acid (250 µmol/L). Consistent with previous findings, conditions of ER stress were associated with significant intracellular lipid accumulation as determined by Oil Red O staining (Supplemental Figure III). Pretreatment of cells with 5 mmol/L valproate or 200 µmol/L of a specific GSK3 inhibitor, GSK3 inhibitor II, attenuated lipid accumulation. This finding indicates that GSK3β activity is required for ER stress–induced lipid accumulation and is consistent with our hypothesis that ER stress signals through GSK3β and that valproate blocks ER stress–induced GSK3β activity.

GSK3β Regulation in Mouse Models of Accelerated Atherosclerosis

To determine the relative effects of hyperglycemia, hyperhomocysteinemia, and high-fat diet on hepatic GSK3β activity, we immunoprecipitated total GSK3β from homogenized liver lysates and monitored its ability to incorporate 32P into a phospho-primed glycogen synthase peptide (p-GS2) substrate. We found that conditions and treatments that increased ER stress levels and accelerated atherosclerosis were associated with significantly enhanced GSK3β activity (Figure 4A). Dietary supplementation with valproate attenuated activation of GSK3β in hyperglycemic, hyperhomocysteinemic, and high-fat–fed mice but had no effect on basal level GSK3β activity in control mice.

GSK3β can be regulated by posttranslational phosphorylation of specific tyrosine and serine residues that can enhance (phospho-Tyr216) or inhibit (phospho-Ser9) GSK3β kinase activity.31,32 We investigated the phosphorylation status of GSK3β by immunoblot analysis of protein isolated...
from mouse liver using antibodies specific for total GSK3β, phospho-Ser9-GSK3β, or phospho-Tyr216 GSK3β. Phosphorylated GSK3β levels were normalized to total GSK3β for each treatment group. Our results show that conditions of hyperglycemia, hyperhomocysteinemia, and high-fat feeding do not significantly affect total GSK3β or phospho-Ser9-GSK3β levels (Figure 4B and 4C). Dietary supplementation with valproate did significantly increase the relative levels of phosphorylation on Ser9 in each of the treatment groups. This finding is consistent with the observed attenuation of GSK3β activity in liver lysates from valproate-supplemented mice (Figure 4A). Hyperglycemia, hyperhomocysteinemia, and high-fat feeding were associated with increased levels of phospho-Tyr216-GSK3β relative to the control groups. Dietary supplementation with valproate appeared to reduce Tyr216 phosphorylation to basal levels in the treatment groups and had no effect on Tyr216 phosphorylation in the control group. Together, these results suggest that GSK3β activity is regulated, at least in part, by ER stress and valproate-induced changes in phosphorylation patterns.

Immunohistochemical staining was performed on fixed cross-sections of aortic root to determine whether the correlation between ER stress and the activation of GSK3β observed in the liver was also present in the developing atherosclerotic lesion. Sections stained with an antibody specific for phospho-Ser9-GSK3β showed increased staining in the vessel wall of mice fed a valproate-supplemented diet relative to mice fed a nonsupplemented standard diet (Figure 5A). The presence of hyperglycemia, hyperhomocysteinemia, or relative dyslipidemia was associated with increased levels of phospho-Tyr216-GSK3β (Figure 5B). Mice receiving valproate supplementation had decreased phospho-Tyr216 staining relative to nonsupplemented controls. Generally, staining was most intense in the macrophage foam cells within the atherosclerotic lesion. Total GSK3β protein levels were not significantly affected by any of the treatments (data not shown). These results suggest that GSK3β, in the liver and the atherosclerotic lesion, is regulated in a similar manner in response to proatherosclerotic stimuli and valproate supplementation.

Discussion

A diverse but finite array of risk factors has been associated with the development of cardiovascular disease. These include dyslipidemia, smoking, stress, diabetes mellitus, hypertension, obesity, and a sedentary lifestyle. Despite considerable advances, our understanding of the molecular mechanisms by which multiple risk factors actively promote atherosclerosis, the underlying cause of myocardial infarction, remains incomplete.

In this study, we investigated the potential role of ER stress signaling and GSK3β activation in the development and progression of atherosclerosis induced by 3 established cardiovascular risk factors. We found elevated levels of diagnostic markers of ER stress and increased GSK3β activity in the livers and atherosclerotic lesions of hyperglycemic, hyperhomocysteinemic, and high-fat-fed mice relative to age matched controls. There was a direct correlation between these changes and the accelerated development of atherosclerosis in each of the mouse models. mice fed a diet supplemented with valproate exhibited ER stress levels similar to those of nonsupplemented controls but had attenuated GSK3β activity and significantly reduced atherosclerotic lesion volume. Together, these data support a role for ER stress–induced GSK3β activity in the accelerated development of hepatic steatosis and atherosclerosis in apoE−/− mice and identify GSK3β as a potential target for antiatherogenic interventions.

Elevated levels of ER stress have previously been observed in mouse models of hyperglycemia, hyperhomocysteinemia, and obesity. The molecular mechanisms by which these conditions promote ER stress and activate the UPR are not fully understood. In cultured cells, both low and high concentrations of glucose have been associated with ER stress, and the glucose metabolite glucosamine is an especially potent ER stress–inducing agent. Alterations in intracellular glucose concentration may disrupt ER homeostasis by interfering with the N-linked glycosylation of proteins, a major factor in proper protein folding; however, there is little direct evidence for this at the present time. Elevated concentrations of homocysteine are associated with ER stress and induction of the UPR in cultured cells, as well as in animal models of hyperhomocysteinemia. It has been suggested that the free thiol of homocysteine can interfere with disulfide bond formation—an essential event in the proper folding of many proteins in the ER. There is also evidence that elevated homocysteine concentration disrupts Ca2+ homeostasis in the ER. The mechanism by which high-fat feeding or the resulting dyslipidemia may promote ER stress is not completely understood. Specific lipids,
including palmitate and unesterified cholesterol, have been shown to induce the UPR in cultured cells, perhaps by directly altering the consistency and function of the ER membrane. More recently, other factors and conditions associated with cardiovascular risk have been shown to promote ER stress, including cigarette smoke and obesity. The association of multiple risk factors with ER stress suggests that activation of ER stress response pathways play a central role in accelerated atherosclerosis. Therefore, it is important to investigate how ER stress activates proatherogenic mechanisms.

The UPR intersects directly and indirectly with many different stress-signaling pathways. There is increasing evidence of the ability of ER stress to signal through GSK3. GSK3 and GSK3 are homologous, ubiquitously expressed serine/threonine kinases that are involved in a variety of intracellular signaling pathways. Dysregulation of GSK3 activity has been implicated in the development of cancer, Alzheimer disease, bipolar disorder, cardiac myopathy, and insulin resistance. We have shown that GSK3-deficient cultured mouse embryonic fibroblasts are protected from glucosamine-induced lipid accumulation and that this effect could be mimicked by pretreatment of wild-type mouse embryonic fibroblasts with valproate or other compounds with GSK3 inhibitory properties. Recently, it has been shown that GSK3 inhibition attenuates ER stress–induced lipid-associated apoptosis in cultured hepatocytes. These findings directly lead to the experiments described in this report that tested the ability of valproate to attenuate accelerated atherosclerosis in apoE−/− mouse models.

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Figure 4. Hepatic glycogen synthase kinase (GSK)-3β activity and phosphorylation in hyperglycemic, hyperhomocysteinemic, and high-fat-diet–fed mice. A, GSK3β was immunoprecipitated from total protein lysates prepared from the livers of control (C), hyperglycemic (HG), high-fat-diet–fed (HF), and hyperhomocysteinemic (HH) mice with or without valproate supplementation, as indicated. GSK3β kinase activity was determined by monitoring 32P incorporation onto a phosho-primed glycogen synthase derived peptide (pG52). Activities are presented relative to unsupplemented control. B, Immunoblot analysis of total protein isolated from C, HG, HF, or HH liver in the presence or absence of valproate supplementation, as indicated. Total protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunostained with antibodies specific for phospho-Ser9 (S9)-GSK3β, phospho-Tyr216 (Tyr216)-GSK3β, total GSK3β, or β actin, as indicated. C, All protein levels were quantified by densitometry, and phospho-S9-GSK3β and phospho-Y216-GSK3β were normalized to total GSK3β and presented relative to the levels found in nonsupplemented control liver. *P<0.05 relative to nonsupplemented control, #P<0.05 relative to nonsupplemented mice from the same treatment group. n=6 to 8 per group.
The hierarchal relationships of these multiple GSK3 regulatory pathways are still being deciphered.

Our findings suggest that atherogenic risk factors induce ER stress, which leads to the activation of GSK3β, in hepatocytes and macrophage foam cells within the atherosclerotic lesion. Our results suggest that conditions of ER stress activate GSK3β by increasing the relative phosphorylation of Tyr216. Furthermore, we show that valproate, in addition to its ability to directly inhibit GSK3β,21 can also alter the phosphorylation status of GSK3β. Specifically, in vivo treatment with valproate is associated with a relative increase in the phosphorylation of Ser9 and decreased phosphorylation of Tyr216. The mechanisms by which this occurs have yet to be determined but likely involve the ability of valproate to interact with upstream kinases or phosphatases. Previous studies have suggested that valproate can affect GSK3β activity by inhibition of Akt.26

Valproate is a potent and commonly prescribed drug that acts both as an anticonvulsant in the treatment of epilepsy and as a mood stabilizer to control bipolar disorder.47 A recently published pharmacoepidemiological study has shown that epileptic patients taking valproate have a significantly reduced risk of myocardial infarction.48 Despite its wide use, the specific molecular mechanism(s) responsible for the clinical efficacy is not known. Exposure to millimolar concentrations of valproate can induce a variety of cellular responses that may be responsible for its clinical effectiveness. In addition to its ability to inhibit GSK3, it has been reported that valproate potentiates GABA-mediated postsynaptic inhibition,49 depletes intracellular inositol concentrations,50 inhibits histone deacetylases,51 and induces the expression of cellular chaperones.52,53 Our data are consistent with a mechanism by which the antiatherogenic effects of valproate are conferred by its ability to inhibit GSK3β activity. In support of this hypothesis, we show that valproate and another, more specific inhibitor of GSK3 attenuate ER stress–induced lipid accumulation in cultured cells. In addition, we have previously shown that valproate protects cultured cells from ER stress–induced apoptosis.53 Together, these findings may explain the reduction in total volume and the decreased necrotic area of atherosclerotic lesions in valproate-supplemented mice. Additional studies, perhaps using GSK3-deficient mice, will be required to further test the role of GSK3 in proatherogenic signaling pathways.

These studies identify a role for ER stress–GSK3 signaling in the progression and development of atherosclerosis. Furthermore, they suggest a mechanism by which pro- and antiatherogenic signals are transmitted via alterations to the phosphorylation status of GSK3β. This is potentially an important step toward our overall understanding of the molecular and cellular mechanisms that lead to cardiovascular disease in that it may provide a new and unexplored class

Figure 5. Aortic glycogen synthase kinase (GSK)-3β and phosphorylation in hyperglycemic, hyperhomocysteinemic, and high-fat-diet–fed mice. Representative sections of aorta sinus from control (C), hyperglycemic (HG), hyperhomocysteinemic (HH), or high-fat-diet–fed (HF) apolipoprotein E–deficient mice, with and without valproate supplementation, as indicated, were immunostained with antibodies specific for phospho-Ser9 (S9)-GSK3β (A) or phospho-Tyr216 (Y216)-GSK3β (B). Arrows indicate positively stained cells. n=5 to 6 per group.
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of targets for the development of antiatherogenic drug therapeutics.

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Disclosures
None.

References
38. Ibrahim SH, Akazawa Y, Canazane SC, Bronk SF, Elmi NA, Wernburg NW, Billadeau DD, Jores GJ. Glycogen synthase kinase-3 (GSK-3)


Endoplasmic Reticulum Stress and Glycogen Synthase Kinase-3β Activation in Apolipoprotein E–Deficient Mouse Models of Accelerated Atherosclerosis
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Supplementary Figure I. Plasma Lipid Profiles. Plasma lipid profiles of control, hyperhomocysteinemic (HH), hyperglycemic (HG), and high fat diet fed (HF) mice with (+V) or without valproate-supplementation. Values represent the average cholesterol concentration in 3 independent plasma samples from each group after plasma fractionation by FPLC.
Supplementary Figure II. The effect of valproate-supplementation on lesion area in the aortas of high fat fed mice. A) Representative mounts of Sudan IV stained aortas of 24 week old C, HF and HF valproate-supplemented mice. B) Quantification of the lesion area as a percentage of aorta surface area. *P<0.05 relative to control mice fed the non-supplemented (control) diet. #P<0.05 relative to the same group fed control diet, n=5-6/group.
Supplementary Figure III. ER stress induced neutral lipid accumulation in HepG2 and the effect of GSK3 inhibition. HepG2 cells were treated with 8μg/ml tunicamycin or 250μM palmitic acid in the presence or absence of 2mM valproate or 200μM GSK3β Inhibitor II for 6 hours. Cells were stained with Oil Red O and imaged. Proportion of stained lipids was quantified using Image J, normalized to the number of cells and presented relative to untreated controls. n=3, *P<0.05 relative to untreated controls, #P<0.05 relative to no inhibitor within same group.