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

Cameron S. McAlpine, Anna J. Bowes, Mohammad I. Khan, Yuanyuan Shi, Geoff H. Werstuck

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<sup>−/−</sup>) mouse models of accelerated atherosclerosis.

Methods and Results—Female apoE<sup>−/−</sup> 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<sup>−/−</sup> mice

Atherosclerosis is a disease of the arterial wall that is characterized by inflammation and lipid accumulation.1 It is the underlying cause of cerebro- and cardiovascular disease that together account for approximately one third of the annual mortality in westernized societies.1,2 Risk factors for cardiovascular disease include diabetes mellitus, hypertension, dyslipidemia, hyperhomocysteinemia, abdominal obesity, smoking, and physical inactivity.3,4 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.5,6 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,7 hyperhomocysteinemia,8 obesity,9 cigarette smoke,10 and elevated levels of unesterified cholesterol11 or palmitate,12,13 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.7,14 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. ER stress–inducing agents also activate nuclear factor-κB, the transcription factor responsible for promoting inflammatory gene expression. In addition, ER stress can activate caspases and promote apoptosis of endothelial cells, macrophages, and other cell types. Together, lipid accumulation, inflammation, and endothelial/macrophase/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. 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. Recently, we have found that dietary supplementation with valproate, a small branch chain fatty acid with GSK3 inhibitory properties, can attenuate accelerated atherosclerosis in a streptozotocin-induced hyperglycemic apoE−/− mouse model. 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 valproate supplementation on vascular and hepatic ER stress and GSK3β activity levels in 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<sup>+/−</sup>) 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. To study the activation of nuclear factor-κB both in vivo and in vitro, we used GSK3β<sup>−/−</sup> mice, purined from the Jackson Laboratory (stock number 093235). 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 mmol/L Tris HCl, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 1% Triton X, 10 mmol/L dithiothreitol, 1 mmol/L benzamidine, 1 mmol/L 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).

**Immunohistochemical Analysis**

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. SPA-891 and SPA-827, 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 Protein G Plus (Pierce). Kinase activity was measured by monitoring the incorporation of 32P onto phospho-glycogen synthase peptide-2 (Upstate Biotech). Briefly, immunoprecipitated GSK3β was combined with 15 μmol/L p-GS-2 and 0.5 μCi/μL [γ<sup>32</sup>P]ATP in a reaction mixture containing 20 mmol/L MOPS, 50 μmol/L EDTA, 0.25 mmol/L Mg acetate, 5 mmol/L MgCl<sub>2</sub>, 5 mmol/L β-glycerol phosphate, 1 mmol/L EGTA, 0.25 mmol/L Na<sub>2</sub>VO<sub>4</sub>, 0.25 mmol/L dithiothreitol, and 35 μmol/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% 0-phosphoric acid and once with acetone. 32P 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. Relative amounts of lipid staining were quantified using ImageJ 1.35 software.

**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 X 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. 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).
We directly compared the effects of chronic hyperglycemia, hyperhomocysteinemia, or high-fat diet on ER stress levels.

### Results

#### Cell Culture

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum at 37°C and 5% CO2. Cells were fixed in 2.5% paraformaldehyde. Images were captured with an Olympus microscope and a 12.5-megapixel DP71 digital camera with IM50 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, GSK3β, 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 μmol/L compared with 4.6±0.8 μmol/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 GSK3β activity both in vitro and in vivo,21,22,23,26.

Plasma valproate concentration in supplemented mice was 33 to 54 μmol/L with the exception of the high-fat-diet mice in which it was significantly lower (7 μmol/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±1.23×10−3 mm3, P<0.05, in hyperglycemic

### 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 mm3</td>
<td>3.80±0.55</td>
<td>3.21±0.56</td>
<td>8.62±1.23†</td>
<td>4.98±0.76†</td>
<td>7.83±0.99†</td>
<td>5.26±0.50†</td>
<td>87.5±1.90</td>
<td>208±9.0†</td>
<td>187±8.0†</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Valproate, μmol/L</td>
<td>0</td>
<td>37.5±14.9</td>
<td>0</td>
<td>33.7±6.2</td>
<td>0</td>
<td>54.2±11.3</td>
<td>0</td>
<td>0</td>
<td>7.3±5.4</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>10.5±2.0</td>
<td>9.4±2.6</td>
<td>23.2±5.6‡</td>
<td>27.0±0.9‡</td>
<td>10.6±0.8</td>
<td>7.9±3.9</td>
<td>8.5±0.3</td>
<td>11.2±0.9*</td>
<td>10.3±0.5*</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>4.6±0.8</td>
<td>5.2±0.8</td>
<td>1.9±1.8</td>
<td>1.2±0.5</td>
<td>14.1±4.0‡</td>
<td>21.2±2.9‡</td>
<td>4.1±1.8</td>
<td>1.7±1.4</td>
<td>1.9±1.0</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>11.6±2.1</td>
<td>13.1±3.7</td>
<td>12.7±3.1</td>
<td>13.5±3.5</td>
<td>15.1±0.6</td>
<td>14.0±1.1</td>
<td>14.8±2.7</td>
<td>25.0±3.6*</td>
<td>19.4±1.8*</td>
</tr>
</tbody>
</table>

n=9 to 16 per treatment group for each measurement. V indicates valproate; HG, hyperglycemic; HH, hyperhomocysteinemic; HF, high fat diet.

*P<0.05 relative to control mice fed the nonsupplemented (control) diet.
†P<0.05 relative to the same group fed the unsupplemented diet.
‡P<0.05 relative to control mice fed the same diet.

For en face staining, descending aortas were collected and periaortietial tissue was removed. Clean aortas were washed in 70% ethanol, stained with 0.5% Sudan IV for 15 minutes, destained with 80% ethanol, and rinsed with dH2O. After longitudinal dissection, stained aortas were mounted en face, and the images were visualized and captured using a Leica surgical microscope and a DC300 digital camera with IM50 software.

Plasma valproate concentration in supplemented mice was 33 to 54 μmol/L with the exception of the high-fat-diet mice in which it was significantly lower (7 μmol/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.
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 lesions from each group were further characterized by measuring necrotic area. Plaque necrosis, defined as acellular regions of the lesion, was quantified and is presented relative to total lesion volume. Hyperglycemic, hyperhomocysteinemic, and high-fat–fed mice had significantly increased levels of necrosis within lesion areas relative to controls (Figure 1C). With valproate supplementation, the proportion of necrosis was significantly reduced in all models (Figure 1C).

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,8,23 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\(^{-/-}\) 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 GSK3\(\beta\) in ER Stress–Induced Lipid Accumulation**

To begin to examine the specific role of GSK3\(\beta\) in lipid accumulation, HepG2 cells were cultured in the presence of ER stress–inducing agents tunicamycin (8 \(\mu\)g/mL) or palmitic acid (250 \(\mu\)mol/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 \(\mu\)mol/L of a specific GSK3\(\beta\) inhibitor, GSK3 inhibitor II, attenuated lipid accumulation. This finding indicates that GSK3\(\beta\) activity is required for ER stress–induced lipid accumulation and is consistent with our hypothesis that ER stress signals through GSK3\(\beta\) and that valproate blocks ER stress–induced GSK3\(\beta\) activity.

**GSK3\(\beta\) Regulation in Mouse Models of Accelerated Atherosclerosis**

To determine the relative effects of hyperglycemia, hyperhomocysteinemia, and high-fat diet on hepatic GSK3\(\beta\) activity, we immunoprecipitated total GSK3\(\beta\) 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 GSK3\(\beta\) activity (Figure 4A). Dietary supplementation with valproate attenuated activation of GSK3\(\beta\) in hyperglycemic, hyperhomocysteinemic, and high-fat–fed mice but had no effect on basal level GSK3\(\beta\) activity in control mice.

**GSK3\(\beta\) can be regulated by posttranslational phosphorylation of specific tyrosine and serine residues that can enhance (phospho-Tyr216) or inhibit (phospho-Ser9) GSK3\(\beta\) kinase activity.\(^31,32\)** We investigated the phosphorylation status of GSK3\(\beta\) by immunoblot analysis of protein isolated from liver homogenates using antibodies specific to phospho-Tyr216 and phospho-Ser9. These analyses showed that conditions of ER stress were associated with increased phosphorylation of GSK3\(\beta\) at both serine and tyrosine residues, indicative of enhanced kinase activity. Pretreatment of cells with valproate significantly reduced phosphorylation of GSK3\(\beta\) at both sites, consistent with our hypothesis that valproate blocks ER stress–induced GSK3\(\beta\) activity.
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 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. 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. This observation is consistent with the observed attenuation of GSK3β 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. 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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 GSK3.18,37,38 GSK3α and β are homologous, ubiquitously expressed serine/threonine kinases that are involved in a variety of intracellular signaling pathways.39 Dysregulation of GSK3 activity has been implicated in the development of cancer, Alzheimer disease, bipolar disorder, cardiac myopathy, and insulin resistance.40 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.22 Recently, it has been shown that GSK3 inhibition attenuates ER stress–induced lipid-associated apoptosis in cultured hepatocytes.41 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.

Intracellular control of GSK3 activity is relatively complex and can be modulated through interactions with a number of different scaffold proteins (axin, adenomatous polyposis coli), as well as intracellular localization.42 Regulation appears to predominantly occur by phosphorylation and dephosphorylation of specific residues. GSK3β activity is inhibited by phosphorylation of an amino-terminal serine residue (Ser9).31 Most evidence suggests that Akt is responsible for phosphorylating Ser9 of GSK3β; however, other kinases may also play a role.43 Activation of GSK3β has been associated with phosphorylation of a specific tyrosine residue (Tyr216).32,44 Lochhead et al have suggested that nascent GSK3β acts as a tyrosine kinase that autophosphorylates Tyr216 during a protein maturation process.45 Other studies have shown that Tyr216 phosphorylation/dephosphorylation is used as a mechanism to activate/inhibit the mature GSK3β

![Figure 4](http://atvb.ahajournals.org/)

**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 phospho-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 (Y216)-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.
protein. 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, 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.

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. A recently published pharmacoepidemiological study has shown that epileptic patients taking valproate have a significantly reduced risk of myocardial infarction. 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, depletes intracellular inositol concentrations, inhibits histone deacetylases, and induces the expression of cellular chaperones. 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. 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.
of targets for the development of antiatherogenic drug therapeutics.

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Disclosures
None.

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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.