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# Evidence Supporting a Role for Endoplasmic Reticulum Stress in the Development of Atherosclerosis in a Hyperglycaemic Mouse Model

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## Abstract

We previously observed a correlation between elevated levels of vascular endoplasmic reticulum (ER) stress and accelerated atherosclerotic plaque development in chronically hyperglycemic apolipoprotein-deficient ($ApoE^{-/-}$) mice. We hypothesize that ER stress plays a causative role in diabetic atherogenesis. Here we examine the temporal relation between the onset of hyperglycemia, glucosamine accumulation in the vessel wall, ER stress, and the development of atherosclerosis. We demonstrate, by using streptozotocin-induced hyperglycemic $ApoE^{-/-}$ mice, that conditions of hyperglycemia increase intracellular glucosamine levels and endothelial ER stress levels in the endothelium before the onset of atherosclerosis. At 15 weeks of age, hyperglycemic mice have significantly larger atherosclerotic lesions ($0.120 \pm 0.023$ *vs.* $0.065 \pm 0.021\,mm^2$; $p = 0.001$) relative to normoglycemic mice. Significantly, hyperglycemia-associated accelerated atherosclerosis is observed before the onset of dyslipidemias, suggesting that leveled glucose is sufficient to promote atherogenesis independently. Diagnostic markers of elevated ER-stress levels are increased in macrophage-derived foam cells in early and advanced atherosclerotic lesions. Dietary supplementation with valproate, a small branched-chain fatty acid that interferes with ER-stress signaling, significantly attenuates accelerated atherogenesis in this model. Together, these data are consistent with a causative role for hyperglycemia-associated ER stress in the development and progression of diabetic atherosclerosis. *Antioxid. Redox Signal.* 11, 2289–2298.

## Introduction

DIABETES MELLITUS is associated with a two- to fourfold increased risk of cardiovascular disease (7, 15). However, the relative contributions of specific characteristics of diabetes, particularly hyperinsulinemia, hyperglycemia, and insulin resistance, to the initiation and progression of vascular disease, and the molecular mechanisms by which these risk factors act, are incompletely understood. This is due, in part, to the complex and interdependent relation between insulin and glucose levels, as well as the frequent concurrence of diabetes with other cardiovascular risk factors including dyslipidemia, obesity, and hypertension (7).

Research from several laboratories, including ours, has identified an association between the development of atherosclerosis and disruptions in endoplasmic reticulum (ER) homeostasis (13, 17, 35, 39). The endoplasmic reticulum is the site of mRNA translation, as well as the folding and modification of proteins destined for secretion or localization to various cellular membrane systems, including the plasma membrane. The ER contains quality-control machinery in the form of protein chaperones, which facilitate the folding of proteins and aid in the removal and degradation of misfolded proteins (5). When the capacity of the ER to process proteins is exceeded by the flux of proteins into the ER, unfolded proteins accumulate in the ER lumen, a condition defined as ER stress. The cellular response to conditions of ER stress is known as the unfolded protein response (UPR). The UPR is a multifaceted cellular self-defense system that restores ER homeostasis by reducing the load of proteins to be folded and increases the concentration of ER-resident chaperones, the folding capacity of the ER, and the degradation of irreversibly misfolded proteins (reviewed in references 27, 30).

The molecular mechanisms that link elevated ER stress levels and the activation of proatherogenic pathways leading to lesion development are not completely understood.

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However, conditions of ER stress have been shown to promote endothelial cell apoptosis (13), lipid biosynthesis and uptake (19, 40), and the expression of inflammatory factors (42). Together these represent the hallmark features of atherosclerosis.

In recent years, it has become apparent that elevated levels of ER stress are directly associated with cardiovascular risk factors including obesity, hyperhomocysteinemia, and diabetes mellitus (25, 39, 43). Furthermore, in mouse models of hyperhomocysteinemia and hyperglycemia, the accelerated development of atherosclerosis correlates with increased levels of diagnostic markers of ER stress in the macrophage/foam cells and vascular smooth muscle cells that make up the atherosclerotic lesion (39, 43). However, despite these recent findings, little is known about the role of ER stress in the initial stages of atherogenesis. Here we begin to examine the causative role of ER stress in atherogenesis by monitoring lesion development, changes in vascular ER stress levels, and changes in plasma lipid levels over the course of the development of atherosclerosis in hyperglycemic and normoglycemic apolipoprotein-deficient ($ApoE^{-/-}$) mice.

## Materials and Methods

### *Cell culture*

The human aortic endothelial cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in EGM-2MV media containing 5.5 m*M* glucose and 5% fetal bovine serum (Lonza, Walkersville, MD). Cells were maintained in a humidified incubator at 37°C with 5% $CO_2$. Glucose, glucosamine, mannitol, and tunicamycin were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). All compounds were prepared fresh in culture medium, sterilized by filtration, and added to the cell cultures. Cell viability was assessed by using Trypan blue exclusion.

### *Immunoblot analysis*

Antibodies to PDI (SPA-891), calreticulin (SPA-600), and the anti-KDEL monoclonal antibody (SPA-827), which recognizes GRP78/BiP, HSP47, and GRP94, were purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). RL2 and CTD110.6 antibodies against O-GlcNAc were purchased from Affinity Bioreagents (Golden, CO) and Convance Research Products Inc. (Emery, CA), respectively. The antibody against the phosphorylated form of PERK (3191) was purchased from Cell Signalling (Danvers, MA). The anti-$\beta$ actin (AC-15) antibody was purchased from Sigma-Aldrich. Normalized concentrations of total protein lysates from cultured cells were solubilized in SDS-PAGE sample buffer and separated on SDS-polyacrylamide gels under reducing conditions. After incubation with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen, Burlington, Ontario, Canada), the membranes were developed by using the SuperSignal chemiluminescent substrate (Pierce Chemical Co., Rockford, IL). Protein bands were quantified by using ImageQuant Version 5.2 (Molecular Dynamics, Sunnyvale, CA).

### *Mouse model of hyperglycemia*

Five-week-old, female ApoE-deficient (B6.129P2-$ApoE^{tm1Unc}$) mice fed a standard chow diet (TD92078; Harlan Teklad, Madison, WI) were divided into two groups. One group ($n = 24$) received 10 intraperitoneal injections with 40 mg/kg body weight streptozotocin (STZ; Sigma-Aldrich) over a 3-week period so that the injections were complete when the mice reached 8 weeks of age. The other group ($n = 24$) received injections with an equal volume of citrate buffer (39). A subset of mice from each group ($n = 8$) was sacrificed at 10, 15, or 25 weeks of age, and plasma and tissues were collected for further analysis. To determine the effects of dietary supplementation with valproate, additional groups of $ApoE^{-/-}$ mice were fed a control diet supplemented with 625 mg/kg sodium valproate (TD02165) from 5 weeks of age until sacrifice at 15 weeks of age. The mice were injected with either citrate buffer or STZ, as described earlier. Plasma valproate concentrations were determined by using an AxSYM system (Abbott Laboratories, Mississauga, Ontario, Canada). All mice had unrestricted access to both food and water throughout the study. The McMaster University Animal Research Ethics Board approved all procedures used in these experiments.

### *Immunohistochemical analysis*

Mice were sacrificed, and hearts were flushed with 1x PBS and perfusion-fixed with 10% neutral buffered formalin. After removal, hearts, including the aortic sinus, were cut transversely and embedded in paraffin. Aortic sinus sections were collected on precoated glass slides for measurement of lesion size (hematoxylin and eosin staining) or immunohistochemical staining (28). The VECTASTAIN ABC System (Vector Laboratories, Burlingame, CA) was used for immunohistochemical staining. Sections were stained with mouse primary antibodies by using appropriate biotinylated secondary antibodies and visualized by using Nova Red. Nonspecific staining was controlled for by using a serial section and preimmune IgG. Images were captured with a CCD color videocamera (Empix Imaging, Mississauga, ON) and analyzed by using Northern Exposure (Empix Imaging) and SigmaScan Pro software.

### *Immunofluorescence analysis*

*En face* preparations of aortae were stained overnight with the KDEL or the RL2 antibody and then incubated for 5 min with the nuclear stain, Sytox green (Invitrogen, Burlington, Ontario, Canada). Images were visualized with a confocal microscope (Yokogawa CSU22 on an Olympus BX61WI base, Calgary, AB) and captured by using a CCD camera (C9300, Hamamatsu).

### *Plasma analysis*

Whole-blood glucose levels were measured by using a DEX glucometer (Bayer, Toronto, Ontario, Canada). Plasma glucose and lipid levels were determined in nonfasted mice by using the colorimetric diagnostic kits for total cholesterol, triglycerides, and glucose purchased from ThermoScientific (Rockford, IL), as previously described (39).

### *Statistical analysis*

Results are presented as the mean $\pm$ standard deviation of at least three independent experiments. Significant differences in treatment groups were determined by using the unpaired

Student's $t$ test. For all analyses, $p < 0.05$ was considered statistically significant.

## Results

### *Elevated concentrations of glucose and glucosamine can induce ER stress in cultured endothelial cells*

Endothelial injury is believed to be the initiating event in the development of an atherosclerotic lesion (22, 31). Here we investigate the effect of elevated concentrations of glucose and glucosamine in endothelial cells. We previously showed that conditions associated with hyperglycaemia induce ER stress in cultured human aortic smooth muscle cells, Thp-1 monocytes and HepG2 cells (39), but not in HEK293 cells (data not shown). HAECs were cultured in the presence or absence of 30 m*M* glucose, 5 m*M* glucosamine or 5 $\mu$g/ml tunicamycin for 8 h. As a control for the osmotic effects of these treatments, cells were exposed to 30 m*M* mannitol. None of these treatment conditions significantly affect cell proliferation or viability (data not shown). Immunoblot analyses of total protein lysates show that exposure to elevated concentrations of glucose or glucosamine resulted in a significant increase in *O*-linked *N*-acetylglucosamine modification of cellular proteins, which is indicative of increased intracellular glucosamine concentration (9) (Fig. 1). Furthermore, these conditions also were observed to increase significantly the levels of diagnostic markers of ER stress (23), including calreticulin and the ER-resident chaperones GRP78/BiP and PDI, in HAECs. This result indicates that conditions associated with hyperglycemia induce ER stress in endothelial cells.

### *The effect of chronic hyperglycemia on metabolic parameters in ApoE$^{-/-}$ mice*

Chronic hyperglycemia was induced by multiple low-dose (40 mg/kg body weight) intraperitoneal injections of STZ, as previously described (17, 29, 39). Blood glucose levels were significantly elevated after the last STZ injection relative to age-matched normoglycemic controls ($11.4 \pm 0.7$ *vs.* $7.4 \pm 0.4$ m*M*; $p < 0.05$) and continued to increase up to 25 weeks of age (Fig. 2A). This observation is consistent with previous reports that multiple low-dose injections of STZ can promote an autoimmune-driven reduction in pancreatic $\beta$–cell mass (4, 12). The chronically hyperglycemic ApoE$^{-/-}$ mice had a significantly lower body weight than the normoglycemic mice at 25 weeks of age ($20.2 \pm 1.4$ *vs.* $23.5 \pm 0.7$ g; $p < 0.05$) (Fig. 2B).

Consistent with our previous observations (39), hyperglycemia-associated dyslipidemia was not evident at 15 weeks of age. However, at 20 and 25 weeks of age, hyperglycemic mice showed significantly elevated levels of total plasma cholesterol (Fig. 2C) and a nonsignificant tendency toward hypertriglyceridemia (Fig. 2D).

### *Accelerated atherosclerosis in hyperglycemic ApoE$^{-/-}$ mice*

We next examined the development and progression of atherosclerosis in normoglycemic and hyperglycemic ApoE$^{-/-}$ mice at 5, 10, 15, and 25 weeks of age. No atherosclerotic lesions were detected in the aortic sinus of 5-week-old ApoE$^{-/-}$ mice (Fig. 3). Ten-week-old mice had early

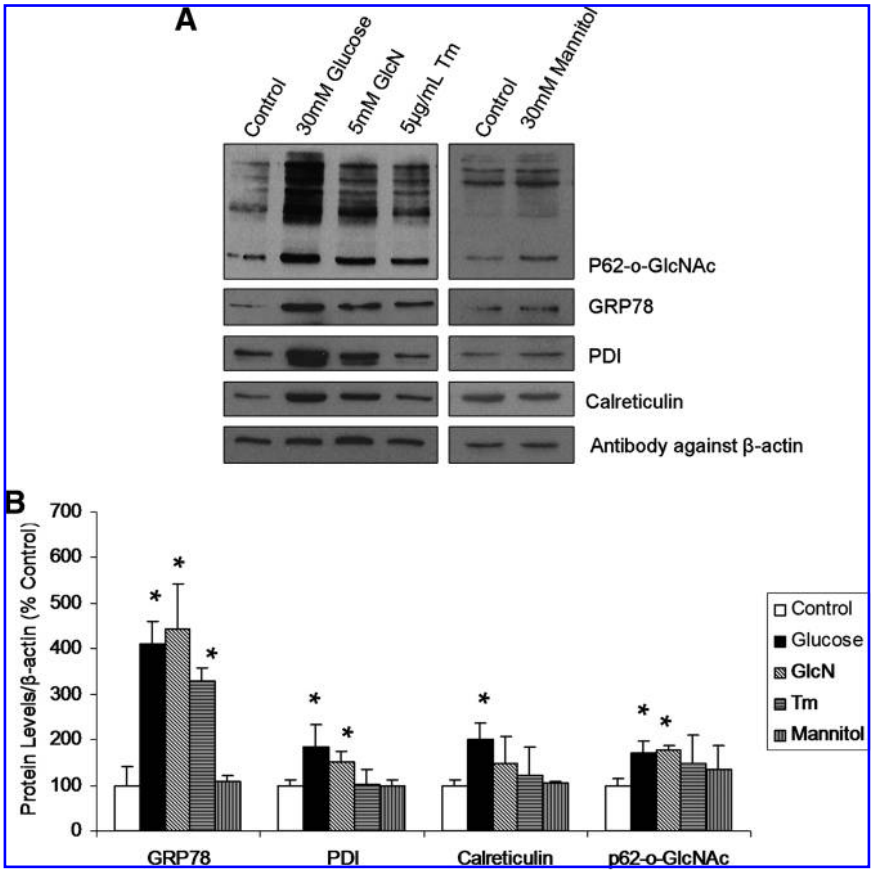


**FIG. 1. Increased O-GlcNAc and ER stress in human aortic endothelial cells cultured in high glucose or glucosamine.** (**A**) HAEC cells were cultured in the absence or presence of the indicated concentrations of glucose, glucosamine (GlcN), tunicamycin (Tm), or mannitol (Man) for 8 h. Total protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunostained by using the RL-2 antibody against *O*-linked GlcNAc or antibodies directed against specific markers of ER stress, including KDEL (GRP78/94), calreticulin, and PDI. As a loading control, an identical blot was immunostained with an antibody against $\beta$–actin. Proteins were visualized with chemiluminescence. (**B**) Protein bands were quantified by using ImageQuant software. The data represent the averages $\pm$ SD, performed in triplicate. *$p < 0.05$ relative to untreated control.

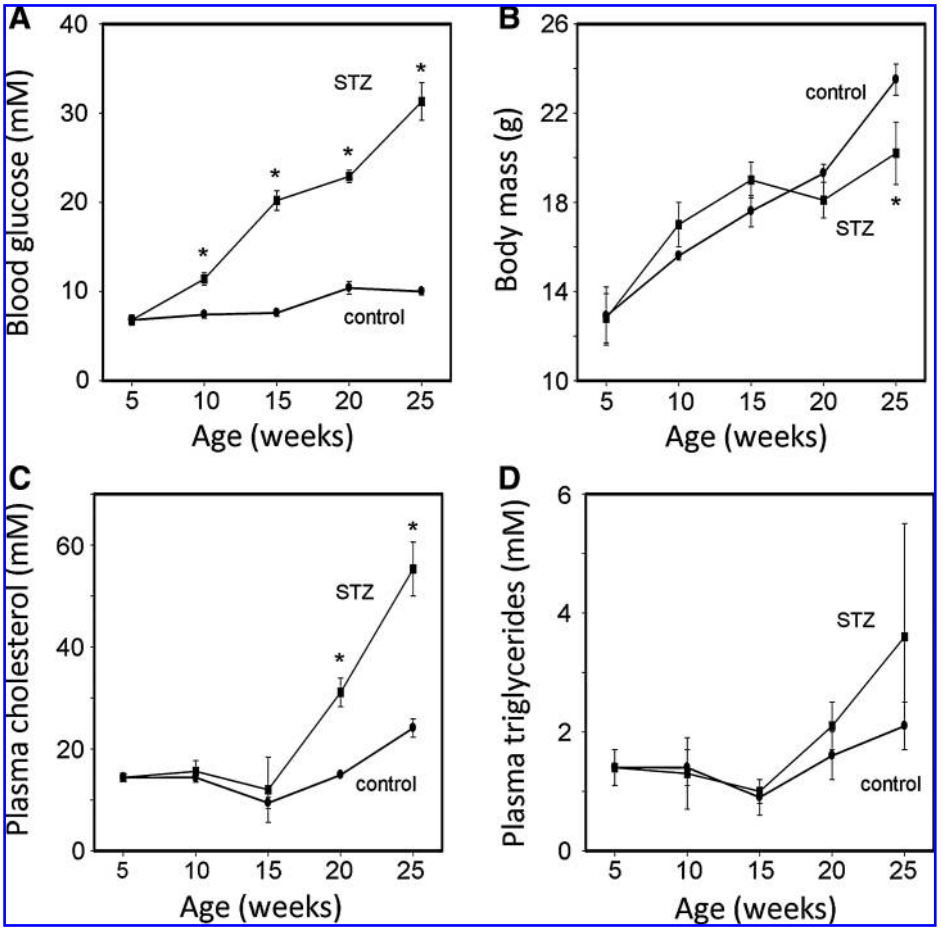


**FIG. 2. The development of chronic hyperglycemia and dyslipidemia in the multiple low-dose–injected ApoE-deficient mouse model.** ApoE$^{-/-}$ mice received 10 intraperitoneal injections of STZ (40 mg/kg body weight) or citrate (equal volume) between 5 and 8 weeks of age. All mice had free access to water and a standard chow diet. At the indicated ages, (**A**) blood glucose, (**B**) body mass, (**C**) total plasma cholesterol, and (**D**) total plasma triglycerides were measured for each group. *$p < 0.05$ relative to corresponding age-matched control; $n = 3$–6/time point/group.

lesions containing macrophage-derived foam cells; however, no significant difference in lesion area was found between normoglycemic and hyperglycemic mice. A significant increase in lesion area and complexity was observed in 15-week-old hyperglycemic mice relative to normoglycemic controls ($0.120 \pm 0.023$ *vs.* $0.065 \pm 0.021$ mm$^2$; $p = 0.001$). At 25 weeks of age, both normoglycemic and chronically hyperglycemic mice had advanced atherosclerotic lesions containing necrotic cores and a fibrous cap. At this time, no significant difference in lesion area was noted between the two groups. No lesions were observed in the descending aortas of either normoglycemic or hyperglycemic mice up to 15 weeks of age. At 25 weeks of age, very small lesions were discernable by Sudan IV staining in the descending aortas of both groups (data not shown).

### *Endothelial ER stress levels are elevated in hyperglycemic ApoE$^{-/-}$ mice before the development of atherosclerosis*

We next examined intracellular glucosamine levels and diagnostic markers of ER stress in the aortas of normoglycemic and hyperglycemic ApoE$^{-/-}$ mice at various stages during the development and progression of atherosclerosis. Sections of aortic sinus from 10-week-old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice were stained with an antibody against proteins containing O-GlcNAc–modified serine and threonine residues. It was previously established that *O*-linked GlcNAc levels are an indicator of intracellular glucosamine concentration (9). We observed increased O-GlcNAc staining in macrophage/foam cells of the atherosclerotic lesions from the hyperglycemic mice relative to normoglycemic controls (Fig. 4).

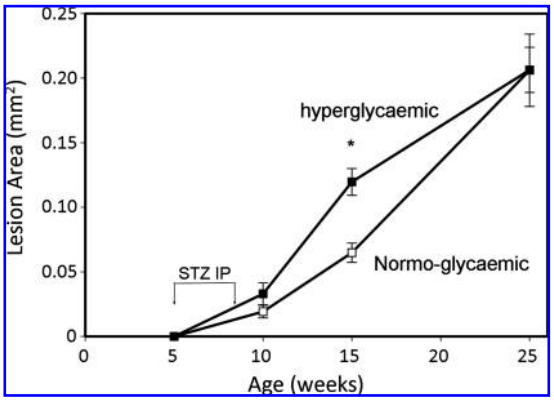


**FIG. 3. Atherosclerotic lesion development in normoglycemic and hyperglycemic ApoE$^{-/-}$ mice.** Cross sections were prepared from the aortic sinus of STZ-injected hyperglycemic and citrate-injected normoglycemic ApoE$^{-/-}$ mice sacrificed at the indicated ages. Aortic sections were stained with hematoxylin and eosin, and lesion areas were determined. *$p = 0.001$ relative to age-matched control; $n = 3$–8 mice/group.

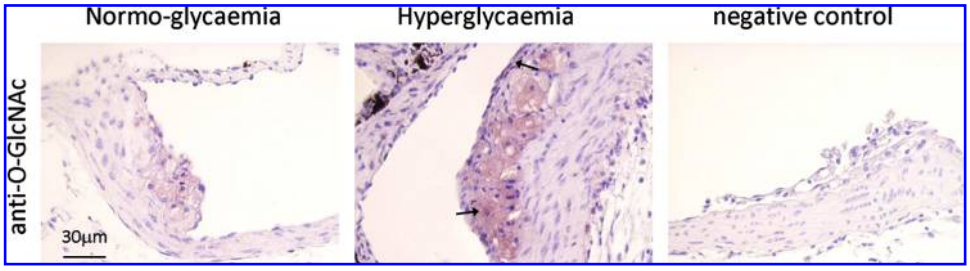


**FIG. 4. Accumulation of O-GlcNAc in the vascular tissue of hyperglycemic ApoE$^{-/-}$ mice.** Aortic cross sections from 10-week-old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice stained with an antibody against proteins containing *O*-linked GlcNAc (CTD110.6).

Cross sections of aortic sinus from 10-, 15-, and 25-week-old normoglycemic and hyperglycemic mice were stained with an anti-KDEL antibody directed against the ER-resident chaperones GRP78/94. Based on the density of staining, increased levels of GRP78/94 are present in the macrophage/foam cells in the lesions of the hyperglycemic mice relative to the normoglycemic mice at each of the times examined (Fig. 5). A similar staining pattern was observed when serial sections of aortic sinus were immunostained with an antibody against the phosphorylated form of PERK, another diagnostic marker of ER stress (43).

*En face* mounts of aortas were prepared from 5-week-old ApoE$^{-/-}$ mice that had not received citrate/STZ injections, and from 10- and 15-week-old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice that had received citrate and STZ-injection, respectively. Aortas were stained with antibodies against O-GlcNAc–modified proteins or KDEL and examined with confocal microscopy (Figs. 6 and 7). In regions of the aortic wall that were free from atherosclerosis, O-GlcNAc staining was localized to the endothelium and was more intense in hyperglycemic mice compared with normoglycemic controls at 10 and 15 weeks of age (Fig. 6). GRP78/94 (KDEL) staining showed a similar pattern (Fig. 7). This ER-stress marker was specifically localized to the endothelial cells, and hyperglycemic mice had relatively more-intense KDEL staining relative to the normoglycemic controls.

### *Dietary supplementation with sodium valproate attenuates accelerated atherosclerosis in hyperglycemic ApoE$^{-/-}$ mice*

Valproate is a small branched-chain fatty acid that has been shown to upregulate ER chaperone levels and protect cells from ER stress–induced dysfunction and cell death (11, 14, 16, 33). Normoglycemic and hyperglycemic ApoE$^{-/-}$ mice were fed a diet supplemented with 625 mg/kg sodium valproate to determine the effects on atherogenesis in this model. Mice fed the supplemented diet had plasma valproate levels of $35.6 \pm 10.6\,\mu M$. At 15 weeks of age, the valproate-supplemented mice were killed, and the lesion area at the aortic sinus was determined (Fig. 8). Lesion areas at the aortic sinus in hyperglycemic ApoE$^{-/-}$ mice fed the valproate-supplemented diet are significantly smaller than lesions in hyperglycemic ApoE$^{-/-}$ mice fed the control diet

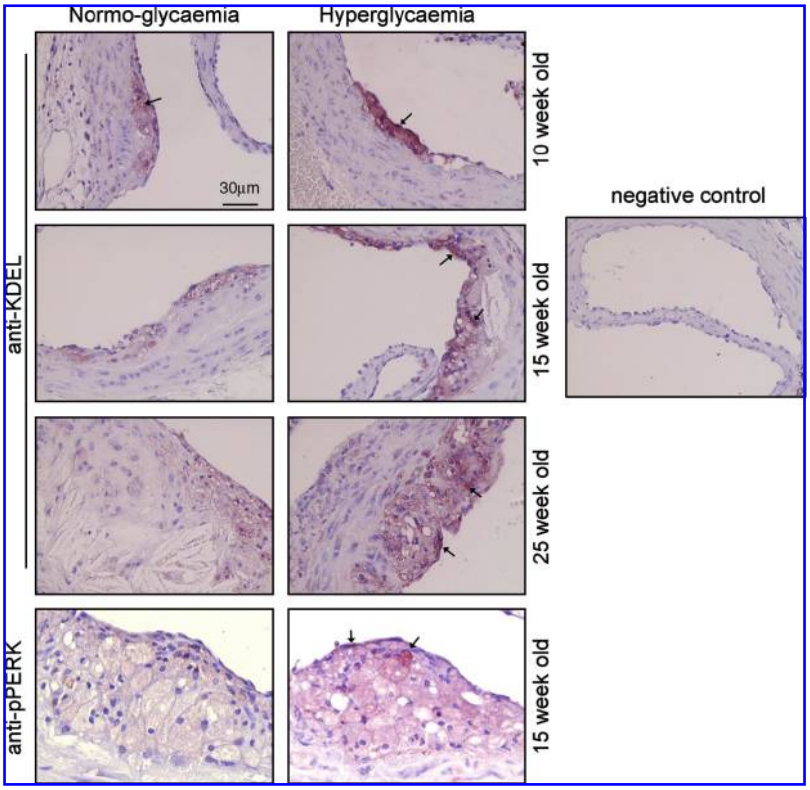


**FIG. 5. Accumulation of O-GlcNAc in the vascular tissue of hyperglycemic ApoE$^{-/-}$ mice.** Aortic sections from 10-, 15-, and 25-week-old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice were stained with the anti-KDEL antibody that detects ER-stress markers GRP78/94 or an antibody against the phosphorylated form of PERK, as indicated. *Arrows* indicate positively stained macrophages, and endothelial and smooth muscle cells. The negative control is a serial aortic section that has been immunostained with preimmune serum.

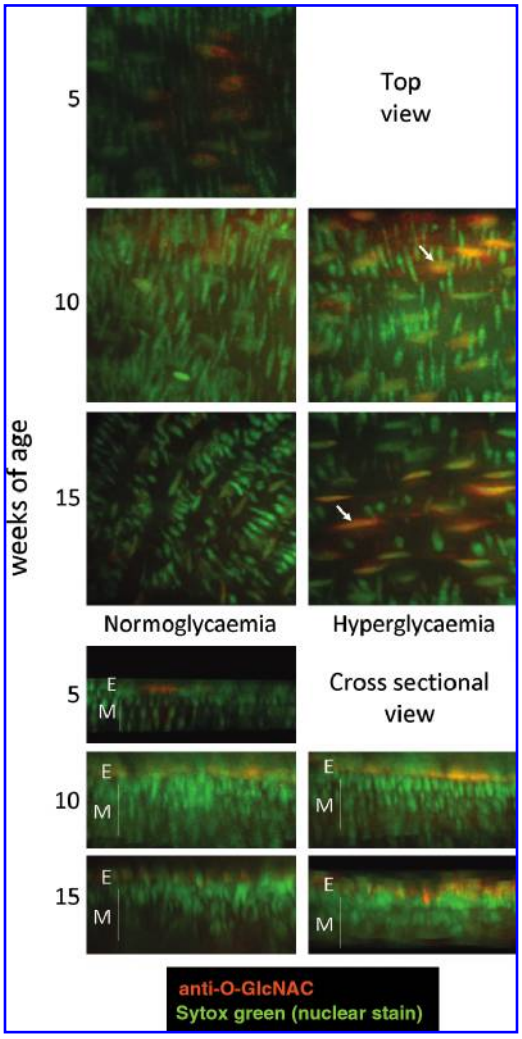


**FIG. 6. Hyperglycemia promotes endothelial *O*-linked GlcNAcylation.** Confocal images of e*n face* aortas from 5-, 10-, and 15-week-old normoglycemic ApoE$^{-/-}$mice and 10- and 15-week STZ-induced hyperglycemic mice were prepared and stained with an antibody against O-GlcNAc (RL2) (red) and counterstained with Sytox green nuclear stain. Images of the endothelial surface (top view) and as well as a cross-sectional view of the vessel wall were captured. *Arrows* indicate endothelial cells. Endothelium (E) and medial (M) layers are labeled.

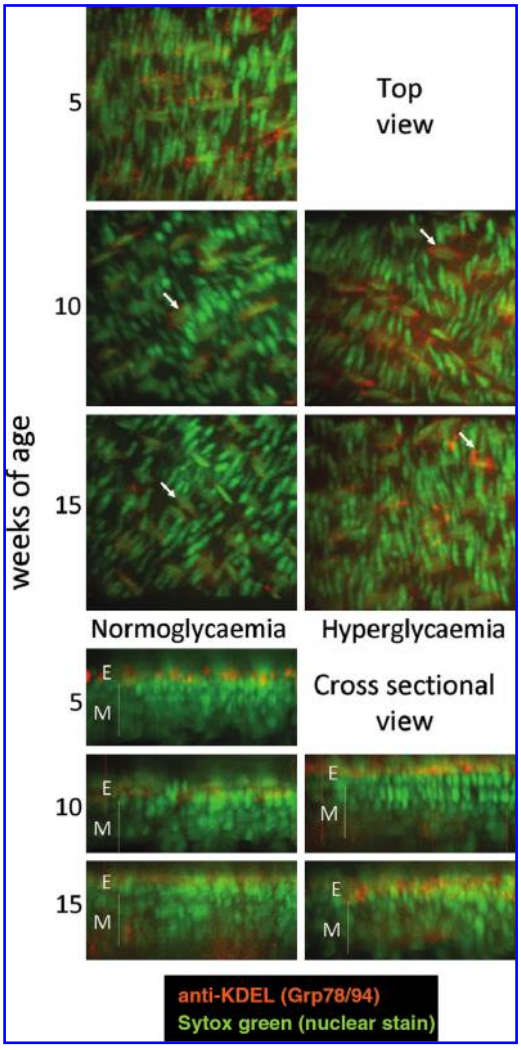


**FIG. 7. Hyperglycemia promotes endothelial ER stress** before **the development of atherosclerosis.** Confocal images of e*n face* aortas from 5-, 10-, and 15-week-old normoglycemic ApoE$^{-/-}$mice and 10- and 15-week STZ-induced hyperglycemic mice were prepared and stained with an antibody against KDEL (GRP78/94) (red) and counterstained with Sytox green nuclear stain. Images of the endothelial surface (top view) and as well as a cross-sectional view of the vessel wall were captured. *Arrows* indicate endothelial cells. Endothelium (E) and medial (M) layers are labeled.

($0.082 \pm 0.034\,mm^2$ *vs.* $0.120 \pm 0.023$; $p < 0.05$). This result is consistent with previous findings from our laboratory (2). The effect of valproate supplementation on normoglycemic mice was not significant relative to controls. Dietary valproate did not significantly affect plasma glucose levels in normoglycemic or hyperglycemic ApoE$^{-/-}$ mice relative to controls.

## Discussion

Diabetes mellitus is associated with a cardiovascular mortality rate that exceeds 70%, and people with diabetes are 2 to 3 times more likely to die of cardiovascular causes than are people with no history of diabetes, even after controlling for other CV risk factors (7, 20, 36). However, the molecular mechanisms and pathways that link diabetes mellitus and hyperglycemia to the activation of proatherogenic processes are not fully understood.

In this report, we investigate the potential role of ER stress in the development of atherosclerosis in an STZ-induced hyperglycemic ApoE$^{-/-}$ mouse model. Our results indicate that hyperglycemia promotes endothelial ER stress in the aorta wall before any morphologic changes in vessel structure or cellular organization. Furthermore, the accelerated development of atherosclerotic lesions, observed in 15-week-old hyperglycemic mice, occurs before the onset of diabetes-associated dyslipidemia. Finally, we show that dietary supplementation with valproate, a small molecule that has been shown to interfere with ER-stress signaling, can attenuate

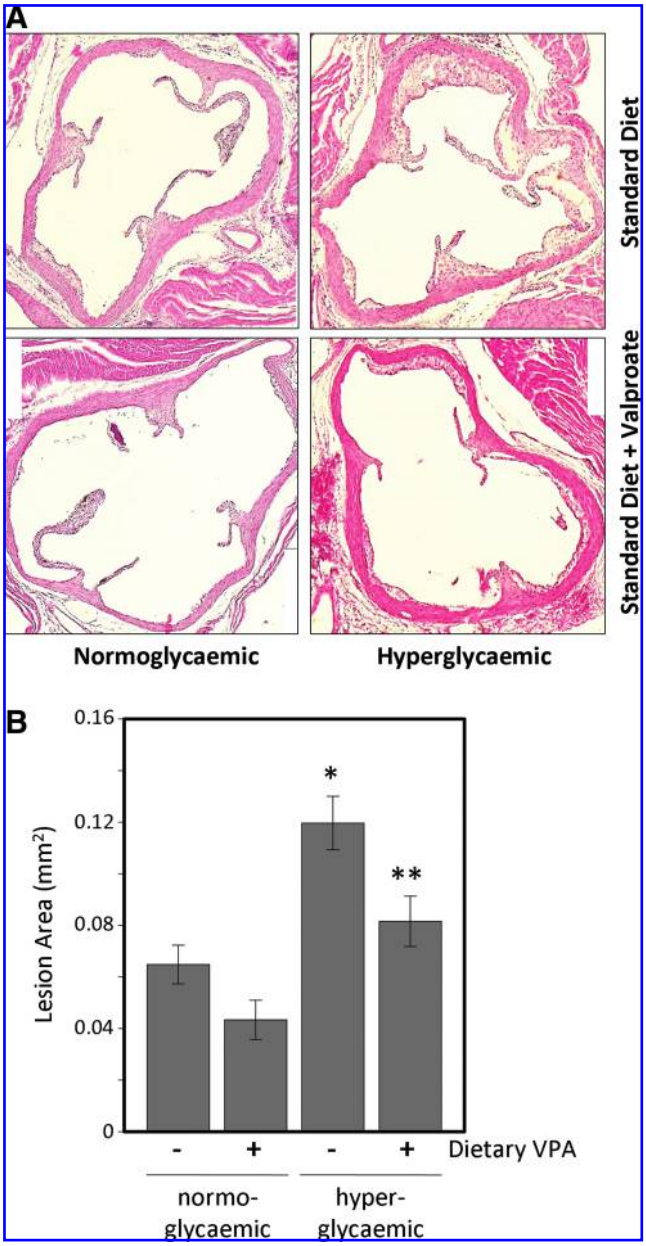


**FIG. 8. Dietary supplementation with valproate attenuates accelerated atherosclerosis in hyperglycemic mice.** (**A**) Hematoxylin and eosin–stained cross sections of aortic sinus from 15-week-old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice fed a standard diet or a diet supplemented with sodium valproate, as indicated. (**B**) Quantification of lesion area at the aortic sinus. *$p < 0.01$ relative to normoglycemic control; **$p < 0.05$ relative to unsupplemented hyperglycemic mice; $n = 8$ mice per group.

accelerated atherosclerosis in hyperglycemic ApoE$^{-/-}$ mice. Together, these findings are consistent with hyperglycemia-induced ER stress playing a causative role in diabetic atherogenesis.

The maintenance of ER homeostasis is known to play an important role in the regulation of blood glucose levels. In the pancreatic $\beta$ cell, impaired UPR function can induce ER stress leading to $\beta$-cell dysfunction and reduced insulin secretion in humans and in mouse models (3, 10, 32). ER stress in the peripheral tissues of ob/ob mice has been shown to link obesity to insulin resistance and type 2 diabetes (25, 26). Our previous findings suggest that exposure to elevated concentrations of glucose ($\sim$30 m*M*) can promote the accumulation of intracellular glucosamine and disrupt ER homeostasis, leading to the activation of the UPR in cultured HepG2, HASMC, and Thp-1 cell lines, as well as in vascular smooth muscle cells and macrophage foam cells in the atherosclerotic lesions of hyperglycemic ApoE$^{-/-}$ mice (16, 39). Here we show that exposure to 30 m*M* glucose or 5 m*M* glucosamine promotes the accumulation of intracellular *O*-linked GlcNAc in HAECs. This effect corresponds to an observed increase in diagnostic markers of ER stress including GRP78, calreticulin, and PDI (Fig. 1).

The mechanistic details of the association between elevated ER-stress levels and the activation of proatherogenic pathways leading to lesion development are not completely understood. However, ER stress has been shown to promote specific cellular effects that may be directly involved in this process. ER stress–inducing agents can activate and dysregulate the sterol regulatory element–binding proteins (SREBPs), transcription factors that control biosynthesis and uptake of triglyceride and cholesterol (19, 40). ER stress–inducing agents, including homocysteine and glucosamine, have been shown to promote the activation of nuclear factor-kappa B (NF-$\kappa$B), the transcription factor that is responsible for promoting the expression of genes involved in inflammatory processes and cell adhesion (16, 41). Finally, ER stress has been shown to induce the activation of caspases and to promote apoptosis in human aortic endothelial cells and hepatocytes (13, 16). Together, the dysregulation of lipid metabolism, the activation of inflammatory pathways, and the induction of cell-specific apoptosis represent the hallmark features of atherosclerosis (22, 31).

A direct correlation between accelerated atherosclerosis and elevated levels of diagnostic markers of ER stress in vascular lesions has been shown in several different mouse models of cardiovascular disease. Specifically, increased protein levels of GRP78/94, CHOP, and the phosphorylated form of PERK have been observed in macrophage foam cells from early and advanced atherosclerotic lesions in hyperhomocysteinemic ApoE$^{-/-}$ mice (42, 43). Similarly, increased levels of GRP78/94 and phosphorylated PERK correlate to the accelerated development of atherosclerosis in chronically hyperglycemic ApoE$^{-/-}$ mice (2, 39). These findings suggest a role for hyperglycemia-induced ER stress in the progression of diabetic atherosclerosis; however, the molecular mechanisms by which ER stress activates proatherosclerotic pathways have yet to be fully elucidated. Furthermore, because these previous investigations have examined the levels of diagnostic markers of ER stress in established atherosclerotic lesions, the potential role of ER stress in the endothelium and the initiation of events leading to the development of early atherosclerotic lesions are not known.

In human patients and in animal models, diabetes and chronic hyperglycemia are often associated with the onset of dyslipidemia (6, 37). Delineation of the molecular and cellular pathways that link diabetes to the accelerated development of atherosclerosis is complicated by the close relation between hyperglycemia and other cardiovascular risk factors including obesity and dyslipidemia (7). In this study,

we monitored the changes in plasma cholesterol, triglyceride, and glucose levels in our hyperglycemic ApoE$^{-/-}$ mice relative to normoglycemic controls, and we correlated these to atherosclerotic lesion development to determine the relative contribution of each of these factors to the observed accelerated lesion development (Figs. 2 and 3). Our data clearly show that, in this multiple low-dose STZ-injection model, chronic hyperglycemia is evident at 10 weeks of age and precedes the development of dyslipidemia, which is not evident until the mice are older than 15 weeks. Furthermore, the significant increase in lesion area at the aortic sinus is observed after the onset of hyperglycemia but before the development of dyslipidemia. At 15 weeks of age, hyperglycemic mice had lesions at the aortic sinus that were approximately twofold larger in area than those in the age-matched normoglycemic controls ($p = 0.001$). At 25 weeks of age, the lesion areas in hyperglycemic and normoglycemic ApoE$^{-/-}$ mice were not significantly different, despite significantly elevated blood glucose and plasma lipid levels. Together, these findings are consistent with a mechanism by which hyperglycemia accelerates early lesion development in a manner that is independent of other factors. At later time points, as the atherosclerotic lesions at the aortic sinus approach a maximal size, lesion development in normoglycemic ApoE$^{-/-}$ catches up to that in the hyperglycemic ApoE$^{-/-}$ mice.

*In vivo*, the accumulation of glucosamine, in the form of *O*-linked GlcNAc, is evident in the lesions of 10-week-old hyperglycemic ApoE$^{-/-}$ mice relative to controls (Fig. 4). ER stress levels are elevated in the lesions of hyperglycemic mice at 10, 15, and 25 weeks of age (Fig. 5). With confocal microscopy of the aortic wall, we found that hyperglycemic ApoE$^{-/-}$ mice have elevated levels of intracellular glucosamine, as well as the ER stress marker, GRP78/GRP94, in the aortic endothelium very early after the onset of hyperglycemia and before the development of a fatty streak, the earliest stage of atherosclerosis (Figs. 6 and 7). These data indicate that hyperglycemia-induced ER stress is localized to the endothelial cells and is not present in the vascular smooth muscle cells of the medial layer. A central concept to our current understanding of atherogenesis is that the initiating event in the development of atherosclerosis is endothelial cell injury (22, 31). Therefore, these findings are consistent with our hypothesis that hyperglycemia-induced ER stress directly promotes the development of atherosclerosis.

Several studies have demonstrated the effectiveness of treatment with small molecules to protect cells from ER stress or the downstream complications of ER stress. 4-Phenylbutyric acid (PBA) is a chemical chaperone that appears to stabilize protein conformation, improve ER folding capacity, and protect cells from agents and conditions associated with ER stress (38). *In vivo* PBA has been shown to inhibit adipogenesis (1) and restore glucose homeostasis (26) and leptin sensitivity (24) in mice exposed to conditions that promote ER stress. Valproate is a short branched-chain fatty acid that increases ER chaperone expression (14, 33, 34) and alleviates downstream complications of ER stress, including apoptosis, lipid accumulation, and activation of inflammatory pathways in cultured cells (2, 11, 16). Like PBA, valproate has been shown to block adipogenesis (18). Here (Fig. 8), and in a previous study (2), we show that dietary supplementation with valproate attenuates accelerated atherogenesis in hyperglycemic ApoE$^{-/-}$ mice. The mechanism by which valproate modulates ER-stress responses and diabetic atherogenesis is not clear; however, our data suggest that these effects could result from its ability to inhibit glycogen synthase kinase (GSK)-3 (2, 16). However, it should be noted that valproate, PBA, and other small molecules that modulate ER-stress responses could also promote changes in cellular metabolism by affecting pathways that are independent of the ER-stress response.

The results presented here support the association of glucosamine-induced ER stress levels and UPR activation with accelerated atherosclerosis in hyperglycemic ApoE$^{-/-}$ mice. Furthermore, the accumulation of glucosamine and diagnostic markers of ER stress, subsequent to the onset of hyperglycemia but before the development of atherosclerosis, is consistent with our hypothesis that hyperglycemia-induced ER stress plays a causative role in atherogenesis.

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## Author Disclosure Statement

The authors have no competing financial interests to disclose.

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**Abbreviations Used**

ApoE$^{-/-}$ = apolipoprotein deficient
CV = cardiovascular
ER = endoplasmic reticulum
GRP = glucose-regulated protein
GSK-3 = glycogen synthase kinase-3
HAECs = human aortic endothelial cells
HASMCs = human aortic smooth muscle cells
HEK293s = human embryonic kidney cells
HepG2 = hepatocellular carcinoma cell line
HRP = horseradish peroxidase
HSP = heat-shock protein
NF-$\kappa$B = nuclear factor-kappa B
O-GlcNAc = *O*-linked *N*-acetyl glycosylation
PBA = 4-phenyl-butyric acid
PBS = phosphate-buffered saline
PDI = protein disulfide isomerase
SDS = sodium dodecylsulfate
SREBPs = sterol regulatory element–binding proteins
STZ = streptozotocin
Thp-1 = human acute monocytic leukemia cell line
UPR = unfolded protein response
VPA = valproate

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