Protein kinase R-like endoplasmic reticulum kinase and glycogen synthase kinase-3α/β regulate foam cell formation

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Abstract  Evidence suggests a causative role for endoplasmic reticulum (ER) stress in the development of atherosclerosis. This study investigated the potential role of glycogen synthase kinase (GSK)-3α/β in proatherogenic ER stress signaling. THP-1-derived macrophages were treated with the ER stress-inducing agents, glucosamine, thapsigargin, or palmitate. Using small-molecule inhibitors of specific unfolded protein response (UPR) signaling pathways, we found that protein kinase R-like ER kinase (PERK), but not inositol requiring enzyme 1 or activating transcription factor 6, is required for the activation of GSK3α/β by ER stress. GSK3α/β inhibition or siRNA-directed knockdown attenuated ER stress-induced expression of distal components of the PERK pathway. Macrophage foam cells within atherosclerotic plaques and isolated macrophages from ApoE−/− mice fed a diet supplemented with the GSK3α/β inhibitor valproate had reduced levels of C/EBP homologous protein (CHOP). GSK3α/β inhibition blocked ER stress-induced lipid accumulation and the upregulation of genes associated with lipid metabolism. In primary mouse macrophages, PERK inhibition blocked ER stress-induced lipid accumulation, whereas constitutively active S9A-GSK3β promoted foam cell formation and CHOP expression, even in cells treated with a PERK inhibitor. These findings suggest that ER stress-PERK-GSK3α/β signaling promotes proatherogenic macrophage lipid accumulation.—McAlpine, C. S., and G. H. Werstuck. Protein kinase R-like endoplasmic reticulum kinase and glycogen synthase kinase-3α/β regulate foam cell formation. J. Lipid Res. 2014. 55: 2320–2333.

Atherosclerosis is an inflammatory disease within the walls of medium and large arteries (1). It is the leading cause of cerebrovascular and cardiovascular diseases, which together account for a third of all deaths in Western societies (1, 2). Multiple risk factors contribute to the initiation and progression of atherosclerosis including diabetes mellitus, hypertension, obesity, dyslipidemia, a sedentary lifestyle, and smoking (3). One of the hallmark features of every stage of atherogenesis, from the fatty streak to the complex plaque, is the presence of lipid-laden macrophages known as foam cells. Intimal macrophage/foam cells accumulate lipids from LDL and modified LDL particles and secrete a variety of inflammatory cytokines. In advanced plaques, foam cells undergo apoptosis, thereby contributing to the formation of a highly thrombotic, lipid rich, necrotic core [reviewed by Moore and Tabas (4)]. The molecular events that promote the initiation and development of atherosclerosis are poorly understood. A better understanding of the signaling networks that regulate foam cell formation and atherosclerotic plaque development may lead to the identification of novel therapeutic targets.

The endoplasmic reticulum (ER) is the organelle responsible for the proper folding, modification, and processing of secretory, transmembrane, and ER resident proteins. If the processing capacity of the ER is overwhelmed, unfolded or misfolded proteins begin to accumulate, a condition known as ER stress. The accumulation of misfolded proteins triggers the initiation of the unfolded protein response (UPR), which is composed of three signaling cascades regulated by ER transmembrane protein kinase R-like endoplasmic reticulum kinase; SREBP, sterol-regulatory element binding protein; VPA, valproate.

Abbreviations: AcLDL, acetylated LDL; ATF, activating transcription factor; ApoE−/−, apoE deficient; CHOP, CCAAT/enhancer binding protein homologous protein; DAPI, 4,6-diamidino-2-phenylindole; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GLN, glucosamine; GRP, glucose-related protein; GSK, glycogen synthase kinase; HFD, high-fat diet; IL, interleukin; IRE, inositol requiring enzyme; KDEL, amino acid sequence Lysine-Aspartic acid-Glutamic acid-Leucine; LDLR, LDL receptor; MOL, multiplicity of infection; NF-κB, nuclear factor κB; PA, palmitic acid; PDI, protein disulphide isomerase; PERK, PKR-like endoplasmic reticulum kinase; SREBP, sterol-regulatory element binding protein; Thaps, thapsigargin; UPR, unfolded protein response; VPA, valproate.

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proteins [reviewed Schroder and Kaufman (5)]. The activation of protein kinase R-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme (IRE) 1, and activating transcription factor (ATF) 6 coordinate the attenuation of protein translation and the increased expression of cellular chaperones, as the cell attempts to reattain ER homeostasis. If these early, adaptive mechanisms are not successful at alleviating the stress, downstream components of the UPR will initiate proapoptotic pathways to eliminate the cell.

ER stress and UPR activation have been associated with the progression and development of atherosclerotic plaques. Multiple cardiovascular risk factors including hyperglycemia (6, 7), hyperhomocysteinemia (7, 8), obesity (9), cigarette smoke (10), and elevated concentrations of unesterified cholesterol (11) or palmitic acid (PA) (12) have each been shown to induce ER stress. ER stress and the activation of the UPR have been observed in atherosclerosis-prone areas of the vessel wall prior to lesion development (13) as well as at all stages of plaque progression (14). Furthermore, the alleviation of ER stress with a chemical chaperone reduces atherosclerotic plaque size in apoE-deficient (ApoE−/−) mice (15). ER stress has also been associated with the dysregulation of lipid metabolism by disruption of sterol-regulatory element binding proteins (SREBPs) (8, 16), the induction of inflammation by nuclear factor κB (NF-κB) upregulation (17, 18), and activation of the proapoptotic process by induction of CCAAT/enhancer binding protein homologous protein (CHOP) expression (19, 20). However, the molecular mechanisms by which ER stress and the UPR activate these and other proatherogenic pathways remain unresolved.

Glycogen synthase kinase (GSK) 3 is a serine/threonine kinase involved in several different cell signaling pathways [reviewed by Doble and Woodgett (21)]. There are two forms of GSK3 encoded on separate genes, a 51 kDa α form and a 47 kDa β form. Regulation of GSK3α/β activity is predominantly, but not entirely, through phosphorylation. Phosphorylation at residue serine 21 of GSK3α/β can be regulated, 24 h later. Recent evidence suggests that the role of GSK3α/β in cell metabolism extends to ER stress and the activation of proatherogenic pathways. In cultured cells, conditions of ER stress activate GSK3β (22, 23). In vivo studies have also demonstrated a role for GSK3α/β in the regulation of NF-κB (24). Furthermore, our group and others have shown that inhibition of GSK3α/β is associated with attenuated atherosclerotic development and reduced hepatic steatosis in different mouse models (7, 30, 31). However, the mechanisms by which ER stress modulates GSK3α and/or β, and how GSK3α/β regulates proatherogenic processes, remain unresolved. In this study, we present evidence showing that ER stress-induced GSK3α/β does not regulate the adaptive components of UPR signaling but instead acts as a modulator of distal, proapoptotic elements of the PERK signaling pathway. Moreover, we demonstrate that inhibition of PERK-GSK3α/β signaling attenuates macrophage lipid biosynthesis and uptake, lipid accumulation, and foam cell formation induced by ER stress.

MATERIALS AND METHODS

Cell culture and treatments

THP-1 human monocytes were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen) containing 10% fetal bovine serum at 37°C and 5% CO2. Monocytes were differentiated into macrophages by exposing the cells to 100 nM PMA for 72 h. Thioglycolate-elicited peritoneal macrophages were isolated from 8-week-old female C57BL6 mice or ApoE−/− mice and cultured in DMEM (Life Technologies) containing 10% fetal bovine serum. Cultured cells were treated with 1 μM thapsigargin (Thaps), 5 mM glutosamine (GLN), or 600 μM PA coupled with 4% BSA for 18 h. Enzymatic activity was inhibited by pretreating the cells with indicated inhibitors and concentrations for 2 h. GSK3α/β activity was inhibited with 4 μM CT99021 (cat#13122, Cayman Chemical). PERK was inhibited with 3 μM GS2606414 (cat#516535, Millipore), IRE1 was inhibited with 6 μM IRE1 Inhibitor III (cat#412512, Millipore), and ATF6 was inhibited with 250 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; cat#A8456, Sigma Aldrich). Adenovirus infections were performed 72 h prior to the experiments using a multiplicity of infection (MOI) of 10 of either an empty adenovirus vector (Ad-CMV-Null) or adenovirus encoding constitutively active GSK3β (Ad-CMV-89A-GSK3β). GSK3α/β siRNA was purchased from Cell Signaling (cat#6014s), and all siRNA experiments were conducted using antibody-free media; 50 nM scramble (control) or GSK3α/β siRNA was transfected into cells using Lipofectamine (cat#16688-019, Invitrogen) for 8 h and then treated, as indicated, 24 h later.

Real-time PCR

Total RNA was isolated from cultured cells using an RNeasy mini kit (Qiagen). RNA was quantified by measuring the absorbance at 260 nm, and RNA purity was verified by calculating the ratio of the absorbance at 260 and 280 nm (A260/A280). cDNA was reverse-transcribed from 1 μg of RNA using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed on the StepOne Plus (Applied Biosystems) using iQ SYBR Green Supermix (Bio-Rad), 1 μg cDNA, and 500 nM forward and reverse primers. See supplementary Table I for primer sequences and amplified product size.

Immunoblot

Total protein lysates were solubilized in kinase buffer containing 50 mM Tris HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton x100, 10 mM DTT, 1 mM benzamidine, 1 mM PMSF, and PhosSTOP Phosphatase Inhibitor (Roche). Protein lysates (15 μg) were diluted in SDS-PAGE gel loading buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Blots were probed with primary antibodies against phospho-S51-EIF2α antibody (cat#3398, Cell Signaling), KDEL (cat#SPA-827, Assay Designs), protein disulfide isomerase (PDI; cat#SPA-891, Assay Designs), CHOP (cat#sc-7351, Santa Cruz Biotechnology), β-catenin (cat#9581, Cell Signaling), GSK3α/β (cat#5676, Cell Signaling), or β-actin (cat#A5854, Sigma-Aldrich). After incubation with the appropriate primary and HRP-conjugated secondary antibodies (Life Technologies), blots were visualized using Chemiluminescence (ECL) Western Blotting Detection Reagent (GE Healthcare) and X-ray film.
Liver and heart, including the aortic root, were removed and embedded in paraffin. Serial sections (4 μm thick) of aortic root were collected on precoated glass slides. Sections were stained with primary antibodies against KDEL, CHOP (cat#sc-575, Santa Cruz), or Mac3 (cat#55322, Becton Dickson Co.). 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. Immunofluorescence was quantified using ImageJ 1.43 software. Briefly, 12 aortic sections from each animal (n = 6 to 7 mice per treatment group), representing the entire length of the lesion, were stained and imaged. Staining intensity above background was determined over a fixed threshold. The staining intensity of the 12 aortic sections from each animal was averaged to provide a staining intensity for each animal. Data shown are average staining intensities for each animal within the group.

**Kinase activity assay**

Total GSK3α/β activity was determined from 250 μg total cell protein (supplementary Fig. I). For isoform-specific analysis, GSK3α or GSK3β were immunoprecipitated from 600 μg total cell protein in kinase buffer using a monoclonal antibody specific for GSK3β (cat#610202, BD Transductions) or GSK3α

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**Fig. 1.** GSK3α/β inhibition does not affect the adaptive UPR. Thp-1-derived macrophages were cultured in the presence or absence of the ER stress-inducing agents Thaps (1 μM), GLN (5 mM), or PA (600 μM) for 18 h. To inhibit GSK3α/β activity, cells were pretreated for 2 h with 4 μM CT99021, a specific GSK3α/β inhibitor. Using quantitative real-time PCR, the expression level of GRP78 (A), GRP94 (B), calreticulin (C), and PDI (D) were determined. n = 3–4, * P < 0.05 relative to untreated cells.
Fig. 2. GSK\(3\alpha/\beta\) is a distal target of the PERK signaling pathway. Thp-1-derived macrophages were treated with 1 \(\mu\)M Thaps, 5 mM GLN, or 600 \(\mu\)M PA in the presence or absence of small-molecule inhibitors of PERK (GSK2606414, 3 \(\mu\)M), IRE1 (IRE1 Inhibitor III, 6 \(\mu\)M), or ATF6 (AEBSF, 250 \(\mu\)M). GSK\(3\alpha/\beta\) activity was determined in whole cell lysates. ER stress-induced GSK\(3\alpha/\beta\) activity, while PERK inhibition, but not IRE1 or ATF6 inhibition, attenuated ER stress-induced GSK\(3\alpha/\beta\) activity (A, \(n = 6-7\)). Thp-1 macrophages were treated with Thaps, GLN, and PA in the presence or absence of the GSK\(3\alpha/\beta\) inhibitor CT99021 (4 \(\mu\)M). Whole cell protein lysates were resolved by SDS-PAGE and probed with antibodies against total or phospho-S51-eIF2\(\alpha\) or CHOP (B). Immunoblots were quantified...
TABLE 1. Metabolic parameters of Apoe<sup>−/−</sup> mice on an HFD with and without VPA supplementation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HFD</th>
<th>HFD + VPA</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>23.4 ± 0.9</td>
<td>23.0 ± 0.5</td>
</tr>
<tr>
<td>Atherosclerotic lesion volume, 10&lt;sup&gt;−3&lt;/sup&gt; mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>208 ± 9.0</td>
<td>187 ± 8.0*</td>
</tr>
<tr>
<td>Necrotic core volume, 10&lt;sup&gt;−3&lt;/sup&gt; mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>132 ± 9.6</td>
<td>97 ± 13.0*</td>
</tr>
<tr>
<td>Plasma VPA, mM</td>
<td>0</td>
<td>7.3 ± 5.4</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>11.2 ± 0.9</td>
<td>10.3 ± 0.5</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
<td>25.0 ± 3.6</td>
<td>19.4 ± 1.8</td>
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<sup>*</sup>n = 9–12 per treatment group.

<sup>p</sup><0.05 relative to control mice fed the nonsupplemented diet.

RESULTS

GSK3α/β inhibition does not affect the adaptive UPR

Thp-1 human monocyte cells were differentiated into macrophages by exposure to 100 nM PMA for 72 h. The small-molecule GSK3α/β inhibitor CT99021 was used to directly inhibit GSK3α/β activity (32). To confirm inhibition, GSK3α and GSK3β were immunoprecipitated from Thp-1 macrophage lysates, and kinase activity was determined in the presence or absence of 0.5 μM CT99021 (35) (supplementary Fig. IIA). GSK3α/β inhibition was verified indirectly by monitoring the accumulation of β-catenin in cells treated with 4 μM CT99021 (supplementary Fig. IIB). To determine the impact of GSK3α/β inhibition on ER stress-induced chaperone expression, macrophages were pretreated for 2 h in the presence or absence of 4 μM CT99021 and then challenged with ER stress-inducing agents, including 1 μM Thaps, 5 mM GLN, or 600 μM PA, for 18 h. Neither ER stress nor GSK3α/β inhibition reduced Thp-1 macrophage cell viability below 80% (supplementary Fig. III). Total RNA was isolated, and quantitative real-time PCR was performed. The expression levels of the cellular chaperones and foldases, glucose-related protein (GRP) 78, GRP94, calreticulin, and PDI, were determined (Fig. 1). These components of the adaptive ER stress response were significantly upregulated by Thaps, GLN, and PA (Fig. 1). GSK3α/β inhibition did not alter GRP78, GRP94, calreticulin, or PDI expression (Fig. 1). Consistent with these findings, siRNA-directed knockdown of GSK3α/β did not alter the ability of Thaps, GLN, or PA to increase GRP78 protein levels (supplementary Fig. IVA–C). These results suggest that GSK3α/β activity is not required for early, adaptive UPR signaling.

GSK3α/β is a target of the PERK signaling pathway

We next investigated the three branches of UPR and the potential role of GSK3α/β in each of these signaling pathways. Initially, the effect of ER stress on GSK3α/β activation was determined. ER stress induced by Thaps, GLN, and PA significantly increased GSK3α/β activity in Thp-1 macrophages (Fig. 2A). Macrophages were then exposed to inhibitors of each of the three UPR signaling pathways. Inhibition of the PERK, but not IRE or ATF6, significantly

by densitometry (C, D). Total RNA was isolated from similarly treated cells and the expression level of ATF4 (E) and CHOP (F) were quantified by real-time PCR. n = 4, * P < 0.05 relative to untreated cells, # P < 0.05 relative to cells of the same treatment without CT99021.
attenuated ER stress-induced GSK3α/β activity (Fig. 2A and supplementary Fig. V). Activated PERK phosphorylates the eukaryotic initiation factor (eIF) 2α at serine 51. This phosphorylation event results in the attenuation of general protein translation and the specific upregulation of ATF4 and CHOP. Immunoblot analysis of protein lysates from macrophages challenged with Thaps, GLN, or PA shows the expected ER stress-induced phosphorylation of
eIF2α, indicative of the activation of the PERK signaling pathway (Fig. 2B, C). P-eIF2α levels were unaffected by GSK3α/β inhibition suggesting that GSK3α/β does not affect PERK activity directly. However, ER stress-induced CHOP and ATF4 expression were blocked by GSK3α/β inhibition and siRNA knockdown (Fig. 2B, D–F, and supplementary Fig. IVA–D). These results indicate that GSK3α/β plays a role in the regulation of downstream components of the PERK branch of the UPR.

GSK3α/β inhibition is associated with attenuated CHOP expression in atherosclerotic macrophages

Having identified a role for GSK3α/β in PERK signaling in vitro, we then asked if CHOP expression in macrophages within the atherosclerotic plaque could be attenuated by GSK3α/β inhibition. Five-week-old female ApoE−/− mice were placed on an HFD containing 21% milk fat and 0.2% cholesterol for 20 weeks. A subset of mice were given an HFD supplemented with VPA (625 mg VPA/kg body weight), a small molecule shown to inhibit GSK3α/β both in vitro and in vivo (7, 30, 34–36). We have previously reported that ApoE−/− mice fed an HFD supplemented with VPA present with attenuated GSK3β activity in hepatic tissue and within the aortic wall and have significantly reduced atherosclerotic plaque volume (by ~10%) and necrotic core area (by ~27%) (Table 1) (7). Aortic sections were stained with appropriate preimmune IgG antibodies to control for nonspecific staining (supplementary Fig. VI). Representative images (Fig. 3A) and quantitation (Fig. 3C) show no detectible alterations to KDEL staining pattern or intensity in the VPA-supplemented mice. Next, serial aortic sections were costained with anti-Mac3 and anti-CHOP antibodies. We observed a significant reduction in CHOP staining within macrophages of the atherosclerotic plaque in VPA-supplemented mice (Fig. 3B, D).

Peritoneal macrophages were isolated from a separate group of ApoE−/− mice fed an HFD or an HFD supplemented with VPA for 3 weeks. Macrophages isolated from mice fed an HFD supplemented with VPA displayed significantly reduced CHOP protein and mRNA expression, while GRP94 and GRP78 mRNA and protein levels were unchanged relative to HFD-fed mice (Fig. 3E–H). These observations are consistent with our in vitro data showing that GSK3α/β does not regulate upstream, adaptive components of the UPR but does modulate distal components of the PERK signaling pathway.

GSK3α/β inhibition attenuates ER stress-induced lipid accumulation in macrophages

We hypothesize that ER stress may play a role in the dysregulated accumulation of lipids in atherosclerotic foam cells. Our data suggest that inhibition of GSK3α/β will modulate this response. To test this, cultured Thp-1 macrophages were challenged with ER stress-inducing agents in the presence or absence of CT99021. After 18 h, the expression levels of selected transcripts involved in lipid biosynthesis and uptake were quantified by real-time PCR. Results show that ER stress was associated with significantly enhanced expression of genes regulating lipid and cholesterol metabolism including FAS, SREBP-1c, SREBP-2, HMG-CoA, and LDL receptor (LDLR) (Fig. 4). Inhibition
Fig. 4. GSK3α/β regulates lipid metabolism in macrophages. ER stress was induced in THP-1 macrophages using 1 μM Thaps, 5 mM GLN, or 600 μM PA treatment with or without GSK3α/β inhibition by 4 μM CT99021. The expression levels of SREBP-1c (A), SREBP-2 (B), HMG-CoA (C), LDLR (D), and FAS (E) were determined by real-time PCR. n = 4, * P < 0.05 relative to untreated cells, # P < 0.05 relative to cells of the same treatment without CT99021.
affected by ER stress or GSK3α/β inhibition (supplementary Fig. VII).

To determine if these changes in gene expression affected lipid and cholesterol accumulation within macrophages, GSK3α/β activity by CT99021 significantly attenuated ER stress-induced FAS, SREBP-1c, SREBP-2, HMG-CoA, and LDLR expression (Fig. 4). Transcript levels of genes involved in other metabolic pathways were not significantly affected by ER stress or GSK3α/β inhibition (supplementary Fig. VII).

To determine if these changes in gene expression affected lipid and cholesterol accumulation within macrophages,
PERK-GSK3 regulates foam cell formation in primary mouse macrophages. Thioglycolate-elicited mouse peritoneal macrophages were isolated from female C57BL/6 mice. Macrophages were exposed to 1 μM Thaps, 5 mM GLN, or 600 μM PA in the presence or absence of PERK inhibitor (3 μM) or CT99021 (4 μM). After treatment, cells were stained with Oil Red O and DAPI. Representative images are shown (A) and quantified (B, C). Primary mouse macrophages were infected with adenovirus encoding constitutively active GSK3β (Ad-S9A-GSK3β) or an empty vector control (Ad-CMV-Null). ER stress was induced in the cells expressing GSK3β-S9A as above in the presence or absence of CT99021 or PERK inhibitor. Cells were stained with Oil Red O and DAPI. Representative images are shown (D) and quantified (E, F). Protein lysates from primary macrophages expressing GSK3β-S9A with or without being exposed to PERK inhibitor were resolved by SDS-PAGE and probed for CHOP and β-actin. Representative blots are shown (G) and quantified (H). CHOP mRNA expression was determined by quantitative RT-PCR in primary macrophages expressing S9A-GSK3β in the presence or absence of PERK inhibitor (I). n = 4–5, *P < 0.05 relative to untreated cells, #P < 0.05 relative to cells of the same treatment without inhibitor.

Esterified and unesterified cholesterol levels were quantified. ER stress induction by Thaps, GLN, and PA resulted in significant accumulation of both esterified and unesterified cholesterol (Fig. 5A, B). GSK3α/β inhibition by CT99021, as well as two other GSK3α/β inhibitors, significantly attenuated the accumulation of free and esterified cholesterol in the macrophages (Fig. 5A, B and supplementary Fig. VIII). Consistent with these findings, Oil Red O staining showed increased lipid droplet formation in the macrophages exposed to ER stress-inducing agents, and siRNA-directed GSK3α/β knockdown attenuated this effect (supplementary Fig. IV).

The mechanism by which ER stress signaling through GSK3α/β promotes macrophage lipid accumulation and foam cell formation could involve altered lipoprotein uptake/efflux and/or altered intracellular lipid biosynthesis. To determine if ER stress signaling through GSK3α/β plays a role in uptake of modified LDL, Thp-1...
macrophages were incubated with acetylated LDL (AcLDL) labeled with Alexa 488. Pretreatment with ER stress-inducing agents enhanced AcLDL uptake, and this effect was blocked by GSK3α/β inhibition (Fig. 5C, D). To examine the effect on lipid biosynthesis, macrophages were treated with ER stress-inducing agents and then cultured in the absence of lipoproteins. ER stress enhanced cellular unesterified cholesterol levels, and this effect was attenuated by GSK3α/β inhibition (supplementary Fig. IX). Neither ER stress nor GSK3α/β inhibition significantly altered macrophage cholesteryl ester levels. Taken together, these data suggest that ER stress signaling through GSK3α/β plays a role in lipid uptake and biosynthesis.

Fig. 6. Continued.
PERK-GSK3α/β signaling regulates ER stress-induced foam cell formation in primary macrophages

To investigate the relevance of PERK-GSK3α/β signaling in primary macrophages, peritoneal macrophages were isolated from C57Bl6 mice. Mouse macrophages were cultured in the presence or absence of ER stress-inducing agents, Thaps, GLN, or PA. Consistent with our findings in Thp-1 cells, ER stress increased intracellular lipid concentrations, as assessed by Oil Red O staining, relative to untreated controls (Fig. 6A–C). Inhibition of PERK or GSK3α/β was sufficient to block ER stress-induced lipid accumulation, and cells treated with either of these inhibitors had lipid concentrations similar to those observed in untreated cells (Fig. 6A–C).

A constitutively active form of GSK3β, S9A-GSK3β, was overexpressed in primary macrophages using an adenovirus vector (Ad-S9A-GSK3β, MOI of 10) (supplementary Fig. X). As a control, cells were infected with an empty adenovirus (Ad-Null, MOI of 10). S9A-GSK3β overexpression resulted in significant lipid accumulation, which was not detectably altered by the presence or absence of ER stress (Fig. 6D–F). PERK inhibition, which blocked ER stress-induced lipid accumulation, and cells treated with either of these inhibitors had lipid concentrations similar to those observed in untreated cells (Fig. 6A–C).

Constitutive GSK3β activation is involved in a number of signaling networks and regulates many aspects of cell metabolism and by guest, on October 22, 2017www.jlr.org Downloaded from http://www.jlr.org/content/suppl/2014/09/02/jlr.M051094.DC1 html Supplemental Material can be found at:

DISCUSSION

Recent evidence suggests a causative role for ER stress in the initiation, development, and progression of atherosclerosis (7, 13, 14, 30). The mechanistic details of how ER stress induces the proatherogenic process are poorly understood. We present data showing that the PERK branch of the UPR signals through GSK3α/β to promote macrophage foam cell formation. Our results suggest that GSK3α/β does not modulate the adaptive components of ER stress signaling including chaperone expression and translation attenuation in human macrophages. Rather, ER stress-induced GSK3α/β activation plays a role in regulating the distal components of the PERK pathway and is involved in the upregulation of transcription factors including ATF4 and CHOP.

Constitutive GSK3β activation is able to overcome the effect of PERK inhibition and promotes lipid accumulation and foam cell formation. Constitutive GSK3β activation also induces CHOP protein and mRNA expression even when PERK is inhibited. Taken together, these results illuminate a novel signaling mechanism by which ER stress may promote lipid accumulation in macrophage foam cells by activation of the PERK-GSK3α/β pathway.

Multiple cardiovascular risk factors are capable of inducing ER stress in cell culture and animal models; however, the mechanisms by which individual risk factors promote ER stress are not fully understood. Elevated levels of ER stress have been observed in animal models of hyperglycemia, obesity, and dyslipidemia (6, 7, 30, 37, 38). In a hyperglycemic state, GLN, a metabolite of glucose, accumulates within cells and acts as a potent inducer of ER stress (39–41). In addition, lipids such as PA and unesterified cholesterol are thought to disturb ER function by disrupting the composition of the ER membrane (11, 42). ER stress/UPR activation may be a central mechanism by which multiple cardiovascular risk factors promote atherosclerosis development.

GSK3α/β is involved in a number of signaling networks and regulates many aspects of cell metabolism and
The involvement of ER stress signaling in metabolic disease has been well established, and the PERK signaling branch of the UPR has been the focus of many studies. In both the ApoE and LDLR knockout mice, CHOP deficiency decreases atherosclerotic plaque size and decreases plaque complexity (46). ApoE−/−CHOP−/− and LDLR−/−CHOP−/− mice fed a Western diet develop atherosclerotic plaques that are less necrotic and display decreased capase-3 activation (46). Similarly, Oyadomari et al. (47) examined a mouse model in which eIF2α phosphorylation was impaired by enhanced growth arrest and DNA damage-inducible protein (GADD) 34 expression. These mice had decreased signaling through the downstream components of the PERK pathway and displayed significantly reduced hepatic fat droplet and triglyceride deposition as well as dramatically improved glucose and insulin tolerance (46). The authors characterized these mice further by observing reduced expression of many genes involved in lipid metabolism including stearoyl-CoA desaturase (SCD)-1, FAS, and Lpl (47). Moreover, ATF4 knockout mice have decreased circulating serum triglycerides and FFAs along with decreased white adipose tissue size and reduced SCD1, FAS, and SREBP-1c expression (48).

While our results show that the IRE1 and ATF6 pathways are not directly involved in ER stress signaling through GSK3α/β, other studies have suggested that the IRE1 and ATF6 do play a role in the regulation of lipid metabolism (49–51). Although poorly defined, cross talk between UPR pathways appears to coordinate the appropriate response to an external stimulus, and thus these pathways may collectively regulate lipid homeostasis (52). These studies, along with the results presented here, suggest that activation of the PERK branch of the UPR leads to increased lipid synthesis and uptake while attenuation of PERK signaling impairs lipid deposition.

Our investigations define an important role for GSK3α/β in the regulation of downstream components of the PERK signaling branch of the UPR. Moreover, PERK-GSK3α/β signaling may play a critical role in the regulation of macrophage lipid accumulation and foam cell formation in vivo. There are a number of important aspects of this pathway that remain to be investigated. For example, it will be important to understand the mechanism by which PERK regulates GSK3α/β and whether other signaling networks such as the PI3K/AKT, Wnt, or MAPK pathways are involved. Second, we need to identify the direct targets of GSK3α/β and understand their roles in the regulation of ATF4 and CHOP expression, as well as lipid metabolism. This will be a challenging task because of the number of putative GSK3α/β substrates that have already been identified (53). It will also be interesting to determine the specific roles of GSK3α and GSK3β in this process. The delineation of these pathways may lead to the identification of novel targets for the development of future antiatherogenic therapeutics. Our work presented here provides an initial step toward a clearer understanding of the mechanisms linking ER stress and proatherogenic processes.

**REFERENCES**


