VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Glycogen Synthase Kinase 3 α Deficiency Attenuates Atherosclerosis and Hepatic Steatosis in High Fat Diet—Fed Low Density Lipoprotein Receptor—Deficient Mice

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Studies have implicated signaling through glycogen synthase kinase (GSK) 3 α / β in the activation of pro-atherogenic pathways and the accelerated development of atherosclerosis. By using a mouse model, we examined the role of GSK3 α in the development and progression of accelerated atherosclerosis. We crossed *Gsk3 α /GSK3 α -knockout* mice with low-density lipoprotein receptor (*Ldlr*) knockout mice. Five-week-old *Ldlr*^{−/−}; *Gsk3 α* ^{+/+}, *Ldlr*^{−/−}; *Gsk3 α* ^{+/-}, and *Ldlr*^{−/−}; *Gsk3 α* ^{−/−} mice were fed a chow diet or a high-fat diet for 10 weeks and then sacrificed. GSK3 α deficiency had no detectable effect on any measured parameters in chow-fed mice. High-fat—diet fed *Ldlr*^{−/−} mice that were deficient for GSK3 α had significantly less hepatic lipid accumulation and smaller atherosclerotic lesions (60% smaller in *Ldlr*^{−/−}; *Gsk3 α* ^{+/-} mice, 80% smaller in *Ldlr*^{−/−}; *Gsk3 α* ^{−/−} mice; *P* < 0.05), compared with *Ldlr*^{−/−}; *Gsk3 α* ^{+/+} controls. GSK3 α deficiency was associated with a significant increase in plasma IL-10 concentration and IL-10 expression in isolated macrophages. A twofold to threefold enhancement in endoplasmic reticulum stress-induced IL-10 expression was observed in Thp-1—derived macrophages that were pretreated with the GSK3 α / β inhibitor CT99021. Together, these results suggest that GSK3 α plays a pro-atherogenic role, possibly by mediating the effects of endoplasmic reticulum stress in the activation of pro-atherogenic pathways. (*Am J Pathol* 2014, 184: 3394–3404; <http://dx.doi.org/10.1016/j.ajpath.2014.07.028>)

Atherosclerosis is a disease of the medium and large arteries that is characterized by inflammation and the accumulation of lipids in the arterial wall. Cardiovascular risk factors, including hyperglycemia, dyslipidemia, obesity, hypertension, and cigarette smoke, promote the accelerated development of atherosclerotic lesions.¹ Atherosclerosis typically originates in regions of the artery that contain bifurcations, branches, or inner curvatures, which tend to disrupt linear blood flow.²

Damaged endothelial cells express cell surface proteins, such as vascular cell adhesion molecule-1 and P-selectin, which mediate the attachment and infiltration of monocytes.³ Intimal monocytes differentiate into macrophages that endocytose low-density lipoprotein (LDL) and modified LDL particles that have entered the intima. This leads to the development of lipid-engorged macrophages, called foam cells, which form fatty streaks in the artery wall. Macrophage foam cells amplify the inflammatory response to vascular

injury by secreting growth factors and cytokines.³ Advanced lesions are characterized by the cytokine-induced migration of vascular smooth muscle cells from the tunica media into the intima. Foam cells ultimately undergo apoptosis, leading to the production of a growing acellular region of the lesion, known as the necrotic core.

Necrosis is believed to be a key feature of unstable plaques that are prone to rupture.⁴ Lesion rupture triggers thrombosis, which can occlude the artery and cause myocardial infarction or stroke. Although great advances have been made in our

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understanding of the cellular processes involved in disease progression, the underlying molecular mechanisms that link these risk factors to pro-atherogenic processes are not clearly understood.

During the past decade, it has become evident that the development and progression of atherosclerosis is associated with impaired protein processing in the endoplasmic reticulum (ER) of vascular cells, a condition known as ER stress.^{5,6} Cardiovascular risk factors, including hyperglycemia, dyslipidemia, obesity, and cigarette smoke, have been associated with elevated levels of ER stress and/or activation of the unfolded protein response (UPR) to ER stress.^{7–10} Elevated levels of UPR proteins, including glucose-regulated protein (GRP)-78 and CCAAT/enhancer-binding protein homologous protein (CHOP), are detected in atherosclerosis-prone regions of the endothelium before the detection of intimal macrophages/foam cells.^{11,12} ER stress and UPR activation are also observed in macrophages/foam cells at all stages of atherosclerotic lesion development. ER stress—inducing agents promote the expression of inflammatory cytokines, the accumulation of lipids, and the initiation of macrophage/foam cell apoptosis.^{5,6,8}

Little is known about the molecular mechanisms that may link ER stress and/or UPR induction to the development of atherosclerosis. We have previously identified a potential role for glycogen synthase kinase (GSK)-3 α and/or β in downstream ER stress signaling.¹³ Conditions of ER stress can enhance GSK3 α/β activity,¹⁴ and dietary supplementation with valproate, a small molecule with GSK3 α/β inhibitory properties, can attenuate accelerated atherogenesis in hyperglycemic, hyperhomocysteinemic, and high-fat—diet (HFD) fed *Apoe*^{−/−} mice.^{15,16} However, the interpretation of these studies is limited by the lack of specificity of valproate as a GSK3 α/β inhibitor, and the inability to distinguish between the respective roles of the α and β forms of GSK3. The kinase domains of GSK3 α/β are 98% homologous, and there is a broad overlap in substrate specificity¹⁷; however, GSK3 α and β are not functionally equivalent, and it is now clear that they have unique and distinct functions, in addition to some overlapping, redundant functions.^{18,19} Although GSK3 β knockout mice are not viable,²⁰ GSK3 α knockout mice exist with little or no overt phenotype.²¹

Herein, we crossed Gsk3 α ^{−/−} mice with low-density lipoprotein receptor—deficient (*Ldlr*^{−/−}) mice to directly and specifically examine the effects of GSK3 α deficiency on HFD-induced atherosclerosis. We monitor effects on ER stress/UPR activation, hepatic lipids, and metabolic parameters, including plasma lipids and body weight. Finally, we examine the effects of GSK3 α deficiency on cytokine expression in isolated peritoneal macrophages.

Materials and Methods

Mouse Models

All animal experiments were conducted with approval of the McMaster University (Hamilton, ON, Canada) Animal

Research Ethics Board. *Ldlr*^{−/−} mice (B6.129S7-*Ldlr*^{tm1Her/J}) were obtained from Jackson Labs (Bar Harbor, ME), and Gsk3 α ^{−/−} mice were a generous gift from Drs. Bradley Doble (McMaster University) and James Woodgett (University of Toronto).^{21,22} Both strains were established in a C57Bl6 genetic background. *Ldlr*^{−/−};Gsk3 α ^{+/+}, *Ldlr*^{−/−};Gsk3 α ^{+/−}, and *Ldlr*^{−/−};Gsk3 α ^{−/−} littermates were identified by PCR genotyping (Supplemental Figure S1) and used in the described experiments.

Five-week-old mice of each genotype were randomly placed on a defined chow diet (model TD92078; Harlan Teklad, Madison, WI) or a HFD containing 21% milk fat and 0.2% cholesterol (42% kcal from fat) (model TD97363; Harlan Teklad). Subsets of mice were given chow or HFD containing 625 mg/kg sodium valproate.²³ All mice had unlimited access to both food and water during the study. Body weights were monitored, and fasted and nonfasted blood glucose and lipids were quantified as previously described.⁷ Lipid levels of nonfasted mice were measured using a colorimetric diagnostic kit for both total cholesterol and total triglycerides (Thermo Scientific, Middletown, VA). Plasma was fractionated using fast-performance liquid chromatography (FPLC) with the FRAC-950 FPLC (GE Life Sciences, Piscataway, NJ), and cholesterol concentration was measured using the infinity cholesterol reagent (Thermo Scientific). Plasma IL-10 was quantified by ELISA (eBioscience Inc., San Diego, CA).

Tissue Collection and Analysis

All mice were anesthetized (isoflurane) and harvested at 15 weeks of age. The vasculature was flushed with 0.9% saline and perfusion fixed with 10% neutral buffer formalin. Hearts, aortas, and liver were collected for further analysis. Serial sections (4 μ m thick) of the aortic root were stained with hematoxylin and eosin (H&E). Images were collected using a Leitz LABORLUX S microscope (Leica Microsystems, Inc., Concord, ON, Canada) connected to a DP71 camera (Olympus Imaging, Center Valley, PA), and lesion area was quantified using ImageJ software version 1.43m (NIH, Bethesda, MD).²⁴ Serial sections were immunostained using the Vectastain avidin-biotin complex system and primary antibodies against GRP78/94 (anti-KDEL; SPA-827; Enzo Life Sciences, Inc., Farmingdale, NY) or CHOP (anti-GADD153; sc-575; Santa Cruz Biotechnology, Santa Cruz, CA), and visualized with the appropriate biotinylated secondary antibody and Nova Red.²⁵ Non-specific staining was controlled for using a similar section and preimmune IgG. Neutral lipid accumulation was determined by Oil Red O staining of sections of frozen liver and aortic sinus. Staining with Sudan IV was used to examine atherosclerosis in *en face*—prepared aortas. All staining was quantified using ImageJ software and normalized to cross-sectional area. Total cholesterol and triglycerides were

Table 1 PCR Primer Sequences

Gene name	Forward	Reverse
Mouse DNA		
<i>LDLR</i>	5'-CCATATGCATCCCCAGTCTT-3'	5'-AATCCATCTTGTTCATGGCCCGATC-3'
<i>Gsk3a</i>	5'-CCCCACCAAGTGATTCTACTGCTA-3'	5'-AACATGAAATCCGGGCTCCAACCTAT-3'
Mouse RNA		
<i>Il1b</i>	5'-CTGCTTCCAAACCTTTGACC-3'	5'-AGCTTCTCCACAGCCACAAT-3'
<i>Il6</i>	5'-ATCCAGTTGCCTTCTTGGGACTGA-3'	5'-TAAGCCTCCGACTTGTGAAGTGGT-3'
<i>Il10</i>	5'-CATGGGTCTTGGGAAGAGAA-3'	5'-AACTGGCCACAGTTTTCAGG-3'
<i>Tnf/TNF-α</i>	5'-AGCCCCCAGTCTGTATCCTT-3'	5'-GAGGCAACCTGACCACTCTC-3'
<i>Hspa5/GRP78</i>	5'-ACCTGGGTGGGAAGACTTT-3'	5'-TCTTCAAATTTGGCCCGAGT-3'
<i>Ddit3/CHOP</i>	5'-TATCTCATCCCCAGGAAACG-3'	5'-CTGCTCCTTCTCCTTCATGC-3'
<i>Actb/β-actin</i>	5'-GGCACCACACCTTCTACAATG-3'	5'-TCTTCAAATTTGGCCCGAGT-3'
Human RNA		
<i>IL1B</i>	5'-GTCAGCTCTCTCCTTTCA-3'	5'-AATGTGGCCGTGGTTTCT-3'
<i>IL6</i>	5'-CCACACAGACAGCCAACTCAC-3'	5'-AGGTTGTTTTCTGCCAGTGC-3'
<i>IL8</i>	5'-CTGTGGAGTTTGGCTGTTTT-3'	5'-CTCCAGTCTTGTCATTG-3'
<i>IL10</i>	5'-GCCTAACATGCTTCGAGATC-3'	5'-TGATGTCTGGGTCTTGGTTC-3'
<i>HSPA5/GRP78</i>	5'-CGAGGAGGAGGACAAGAAGG-3'	5'-CACCTGAACGGCAAGAACT-3'
<i>DDIT3/CHOP</i>	5'-TGCCTTTCTCTCGGACACT-3'	5'-TGTGACCTCTGCTGTTCTG-3'
<i>ACTB/β-actin</i>	5'-ACCGAGCGCGCTACAG-3'	5'-CTTAATGTGACGCACGATTTCC-3'

determined in liver lysates using colorimetric diagnostic kits (Thermo Scientific).

Immunoblot Analysis

Total protein lysates were prepared from frozen mouse liver solubilized in 4× SDS-PAGE sample buffer [0.5 mol/L Tris-HCl (pH 6.8), glycerol, and 10% SDS] and quantified by Bradford assay. Samples were diluted in SDS-PAGE loading buffer to a protein concentration of 4 μg/μL. Total protein liver lysates were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Membranes were stained with antibodies against GSK3α (number 07-389; Cell Signaling, Beverly, MA), GSK3β (number 610202; BD Transduction Laboratories, Mississauga, ON, Canada), or β-actin (number A3854; Sigma-Aldrich, Oakville, ON,

Canada) as the loading control. Membranes were incubated for 1 hour with the appropriate horseradish peroxidase secondary antibody (Invitrogen Life Technologies, Inc., Burlington, ON, Canada) and developed using Immobilon Western chemiluminescent horseradish peroxidase substrate (EMD Millipore, Etobicoke, ON, Canada).

GSK3α and -β Activity Assays

Total protein lysates were prepared from livers or isolated macrophages from *Ldlr*^{-/-}; *Gsk3a*^{+/+}, *Ldlr*^{-/-}; *Gsk3a*^{+/-}, and *Ldlr*^{-/-}; *Gsk3a*^{-/-} mice. GSK3 activity was measured by monitoring the incorporation of ³²P onto phospho-glycogen synthase peptide-2 (Upstate, Billerica, MA), as previously described.¹⁵ Kinase activity was determined in total liver extracts and on immunoprecipitated GSK3α and GSK3β.

Table 2 Metabolic Parameters of Valproate-Supplemented *Ldlr*^{-/-} Mice

Parameters	Chow diet		HFD	
	Without valproate	With valproate	Without valproate	With valproate
Body weight (g)	20.20 ± 0.10	21.60 ± 0.30	21.40 ± 0.46	21.96 ± 0.16
Liver weight (g)	0.84 ± 0.10	0.83 ± 0.03	1.32 ± 0.09*	1.10 ± 0.06*
Fat pad weight (g)	0.35 ± 0.06	0.29 ± 0.03	0.52 ± 0.11*	0.47 ± 0.10*
NFBG (mmol/L)	10.00 ± 1.70	8.40 ± 1.20	7.30 ± 2.20	7.90 ± 0.40
Cholesterol (mmol/L)	6.32 ± 0.67	4.42 ± 0.51	15.87 ± 1.11*	17.67 ± 4.47*
Triglyceride (mmol/L)	0.02 ± 0.25	0.01 ± 0.01	0.58 ± 0.22*	0.89 ± 0.28*
Lesion volume (mm ³)	0.11 ± 0.10	0.10 ± 0.09	10.0 ± 3.2*	5.7 ± 3.8*†
Necrotic volume (mm ³)	0	0	3.6 ± 1.5*	1.5 ± 1.1*†

n = 5 to 10 per group.

**P* < 0.05 relative to age-matched chow-fed mice.

†*P* < 0.05 relative to age-matched HFD (valproate)-fed mice.

HFD, high fat diet; NFBG, nonfasting blood glucose.

Figure 1 Atherosclerosis is attenuated in high fat diet (HFD)-fed *Ldlr*^{-/-} mice supplemented with valproate. **A:** Representative hematoxylin and eosin-stained cross sections of the aortic sinus from 15-week-old HFD-fed *Ldlr*^{-/-} mice supplemented with valproate. Atherosclerotic lesions are outlined. **B:** Quantification of lesion (gray bars) and necrotic core (black bars) volume in the ascending aorta of 15-week-old *Ldlr*^{-/-} mice fed a standard chow diet ($n = 4$) or a HFD ($n = 9$) supplemented with and without valproate, as indicated. * $P < 0.05$ relative to corresponding chow-fed controls; † $P < 0.05$ relative to nonsupplemented mice.

Lipid Staining

Neutral lipid accumulation was determined by Oil Red O staining of sections of frozen liver and aorta. Cryosections (8 μ m thick) were collected on precoated glass slides and fixed in 37% formaldehyde for 10 minutes. Sections were stained with a 0.5% w/v stock solution of Oil Red O dissolved in 100% isopropanol and diluted in double-distilled water to a 60% working solution. Nuclei were stained with Meyer's hematoxylin

solution (MHS32; Sigma-Aldrich), and slides were mounted in Crystal-mount media. Lipid staining was quantified using ImageJ software and normalized to cross-sectional area.

Macrophage Isolation and Analysis

Thioglycolate-elicited peritoneal macrophages were isolated from HFD-fed 15-week-old *Ldlr*^{-/-}; *Gsk3a*^{+/-}, *Ldlr*^{-/-}; *Gsk3a*^{+/-}, and *Ldlr*^{-/-}; *Gsk3a*^{-/-} mice and cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies) containing 10% fetal bovine serum. Thp-1 monocytes were differentiated into macrophages in 100 nmol/L phorbol myristate acetate (Sigma-Aldrich). Cells were treated with 2.5 to 4 μ g/mL tunicamycin, 5 mmol/L glucosamine, or 600 μ mol/L palmitic acid for 18 hours. Transcripts encoding ER stress markers and inflammatory cytokines were quantified by RT-PCR using primers described in Table 1.²⁶

Gene Expression Analysis

Total RNA was extracted from peritoneal macrophages isolated from *Ldlr*^{-/-}; *Gsk3a*^{+/-}, *Ldlr*^{-/-}; *Gsk3a*^{+/-}, and *Ldlr*^{-/-}; *Gsk3a*^{-/-} mice using the RNeasy Mini Kit (catalog number 74,104; Qiagen, Inc., Toronto, ON, Canada). RNA concentration/purity was determined by measuring the absorbance at 260/280 nm. RT-PCR was performed using 2 μ g of total RNA, which was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Invitrogen Life Technologies) following the manufacturer's guidelines. Amplification of PCR product was performed using the Mastercycler gradient thermocycler (Eppendorf, Mississauga, ON, Canada). RT-PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems, Foster City, CA) and SYBR GreenER qPCR SuperMix (Invitrogen Life Technologies). All PCRs were performed in biological and technical triplicates to obtain gene expression profiles using primers for the selected inflammatory cytokines (Table 1). Results from RT-PCR were analyzed using Data Assist software version 3.0 (Invitrogen Life Technologies).

Table 3 Metabolic Parameters of *Ldlr*^{-/-}; *Gsk3a*-Deficient Mice

Parameters	<i>Ldlr</i> ^{-/-} ; <i>Gsk3a</i> ^{+/-}		<i>Ldlr</i> ^{-/-} ; <i>Gsk3a</i> ^{+/-}		<i>Ldlr</i> ^{-/-} ; <i>Gsk3a</i> ^{-/-}	
	Chow	HFD	Chow	HFD	Chow	HFD
Body weight (g)	21.0 $\pm$ 0.6	23.6 $\pm$ 0.5*	18.8 $\pm$ 0.7	20.9 $\pm$ 0.5*	20.2 $\pm$ 0.5	23.1 $\pm$ 0.6*
Liver weight (g)	0.84 $\pm$ 0.05	1.17 $\pm$ 0.03*	0.83 $\pm$ 0.03	1.08 $\pm$ 0.07*	0.87 $\pm$ 0.03	1.11 $\pm$ 0.04*
Fat pad (g)	0.14 $\pm$ 0.02	0.28 $\pm$ 0.05*	0.13 $\pm$ 0.02	0.25 $\pm$ 0.06*	0.09 $\pm$ 0.01	0.21 $\pm$ 0.04*
FBG (mmol/L)	7.31 $\pm$ 0.38	7.29 $\pm$ 0.29	8.03 $\pm$ 0.87	7.30 $\pm$ 0.40	7.35 $\pm$ 0.55	8.33 $\pm$ 0.61
NFBG (mmol/L)	8.85 $\pm$ 0.37	7.46 $\pm$ 0.31	7.43 $\pm$ 0.33	8.42 $\pm$ 0.34	7.92 $\pm$ 0.27	7.06 $\pm$ 0.40
Cholesterol (mmol/L)	6.66 $\pm$ 0.84	20.11 $\pm$ 0.15*	8.31 $\pm$ 1.86	17.78 $\pm$ 2.74*	6.86 $\pm$ 0.31	19.95 $\pm$ 0.13*
Triglyceride (mmol/L)	0.92 $\pm$ 0.07	4.78 $\pm$ 0.54*	0.94 $\pm$ 0.12	4.68 $\pm$ 1.21*	1.06 $\pm$ 0.22	3.89 $\pm$ 0.31*
Lesion volume (10 ³ mm ³)	ND	80 $\pm$ 20*	ND	32 $\pm$ 5.0*†	ND	20 $\pm$ 2.0*†
Necrotic volume (10 ³ mm ³)	ND	26 $\pm$ 6.0*	ND	9 $\pm$ 2*†	ND	3 $\pm$ 1*†

$n = 5$ to 10 per group.

* $P < 0.05$ relative to age-matched chow-fed mice of the same genotype.

† $P < 0.05$ relative to age-matched HFD-fed *Ldlr*^{-/-}; *Gsk3a*^{+/-} mice.

HFD, high fat diet; FBG, fasting blood glucose; NFBG, non-fasting blood glucose; ND, not detected.

Figure 2 Glycogen synthase kinase 3 α deficiency significantly attenuates atherosclerosis in high fat diet (HFD)-fed *Ldlr*^{-/-} mice. **A:** Representative cross sections of aortic sinus from 15-week-old HFD-fed female *Ldlr*^{-/-};*Gsk3a*^{+/+}, *Ldlr*^{-/-};*Gsk3a*^{+/-}, and *Ldlr*^{-/-};*Gsk3a*^{-/-} mice stained with Oil Red O. Lesions are indicated by arrowheads. **B:** Quantification of the lesion area in the ascending aorta. **C:** Quantification of the lesion and necrotic core volume in the ascending aorta. **D:** Representative images of cross sections of the aortic sinus from *Ldlr*^{-/-};*Gsk3a*^{+/+} and *Ldlr*^{-/-};*Gsk3a*^{-/-} mice analyzed by immunofluorescence microscopy. Sections were stained with antibodies specific for macrophages/foam cells (anti-F4/80; red) or vascular smooth muscle cells (anti- α -actin; green). Immunostained areas were quantified and normalized to total lesion area. $n = 8$ to 9 per group (**B** and **C**); $n = 4$ per group (**D**). $^{\dagger}P < 0.05$ relative to *Gsk3a*^{+/+} mice.

Statistical Analysis

All *in vitro* experiments are representative of at least three independent experiments. The numbers of mice in each experimental group are indicated. All data are expressed as means $\pm$ SD. An unpaired Student's *t*-test or one-way analysis of variance test was used, as appropriate, to determine statistical significance. $P < 0.05$ was considered statistically significant.

Results

Dietary Supplementation with Valproate Attenuates Atherosclerosis in HFD-Fed *Ldlr*^{-/-} Mice

We have previously shown that dietary supplementation with valproate (625 mg valproate/kg body weight), a compound with GSK3 α / β inhibitory properties, can attenuate accelerated

atherogenesis in hyperglycemic, hyperhomocysteinemic, and HFD-fed *Apoe*^{-/-} mice.^{16,23} To examine the anti-atherogenic potential of valproate in a different mouse strain, we supplemented the diets (standard chow or HFD) of 5-week-old female *Ldlr*^{-/-} mice with 625 mg valproate/kg body weight. At 15 weeks of age, all mice were sacrificed and analyzed. Valproate supplementation had no significant effect on body weight, nonfasting blood glucose, or plasma lipid levels relative to mice fed nonsupplemented diets (Table 2). No significant atherosclerosis was detected at the aortic sinus or ascending aorta of chow-fed *Ldlr*^{-/-} mice, in the presence or absence of valproate supplementation. As expected, the HFD-fed mice had significantly increased liver weight and plasma lipid levels relative to chow-fed mice; however, total body weight was not significantly different (Table 2). Mice fed a HFD developed large advanced atherosclerotic lesions in the aortic sinus and ascending aorta by 15 weeks of age (Figure 1). Mice fed a valproate-supplemented HFD had significantly

Figure 3 Glycogen synthase kinase 3 α deficiency reduces high fat diet (HFD)-induced hepatic lipid accumulation. **A:** Representative images of liver sections, from chow- and HFD-fed *Ldlr*^{-/-}; *Gsk3a*^{+/+}, *Ldlr*^{-/-}; *Gsk3a*^{+/-}, and *Ldlr*^{-/-}; *Gsk3a*^{-/-} mice stained with Oil Red O. Quantification of hepatic total cholesterol (**B**) and triglycerides (**C**) in lysates from chow- (black bars) and HFD-fed (gray bars) GSK3 α -deficient mice. $n = 3$ to 5 per group. * $P < 0.05$ relative to chow-fed mice of the same genotype; † $P < 0.05$ relative to *Gsk3a*^{+/+} mice fed the same diet.

smaller lesions ($5.7 \pm 3.8 \text{ mm}^3$ versus $10.0 \pm 3.2 \text{ mm}^3$; $P < 0.05$) containing smaller necrotic cores ($1.5 \pm 1.1 \text{ mm}^3$ versus $3.6 \pm 1.5 \text{ mm}^3$; $P < 0.05$) compared with mice fed a nonsupplemented HFD. These results indicate that valproate supplementation can attenuate atherogenesis in *Ldlr*^{-/-} mice.

Ldlr/*Gsk3a* Double-Knockout Mice Have No Overt Phenotype on a Standard Chow Diet

Evidence from our previous *in vitro* and *in vivo* investigations has suggested that valproate attenuates atherogenesis by inhibiting GSK3 α and/or β .^{16,23} However, valproate is a non-specific drug that can affect many other metabolic pathways in addition to GSK3 α/β .²⁷ To specifically examine the potential role of GSK3 α in the progression and development of atherosclerosis, we crossed GSK3 α -deficient mice with atherosclerosis-prone *Ldlr*^{-/-} mice to generate *Ldlr*^{-/-}; *Gsk3a*^{+/-} and *Ldlr*^{-/-}; *Gsk3a*^{-/-} genotypes (Supplemental Figure S1). *Ldlr*^{-/-}; *Gsk3a*^{+/-} and *Ldlr*^{-/-}; *Gsk3a*^{-/-} mice were born at expected mendelian frequencies and do not display any overt developmental phenotype. Total hepatic GSK3 α/β activity was assessed using an established kinase assay and found to be reduced by approximately 20% in GSK3 α -deficient mice (Supplemental Figure S2). GSK3 α -specific activity was reduced 50% and 100% in *Gsk3a*^{+/-} and *Gsk3a*^{-/-} mice,

respectively. In isolated peritoneal macrophages, GSK3 β activity was not significantly affected by GSK3 α deficiency.

GSK3 α deficiency did not detectably alter total body weight, liver or fat pad weight, fasting or nonfasting blood glucose, response to an oral glucose tolerance test, or plasma lipid levels in *Ldlr*^{-/-} mice (Table 3 and Supplemental Figure S3). GSK3 α deficiency had no detectable effect on the aortic sinus in chow-fed *Ldlr*^{+/+} or *Ldlr*^{-/-} mice (Supplemental Figure S4). No atherosclerotic lesions were detected in any of the chow-fed mice at 15 weeks of age. Together, these findings are consistent with previous reports indicating that GSK3 α deficiency is not associated with any overt phenotype in mice with a C57Bl6 genetic background.²²

Ldlr/*Gsk3a* Double-Knockout Mice Have Attenuated HFD-Induced Accelerated Atherosclerosis

To determine the effect of GSK3 α deficiency on atherosclerosis, *Ldlr*^{-/-}; *Gsk3a*^{+/+}, *Ldlr*^{-/-}; *Gsk3a*^{+/-}, and *Ldlr*^{-/-}; *Gsk3a*^{-/-} mice were fed a HFD. HFD significantly increased liver and fat pad weight as well as plasma lipid concentrations to a similar extent in all mouse lines (Table 3). GSK3 α deficiency had no detectable effect on these, or any other, metabolic parameters that were assessed. However, *Ldlr*^{-/-}; *Gsk3a*^{-/-} and *Gsk3a*^{+/-} mice had significantly smaller, less advanced atherosclerotic lesions in the aortic sinus and ascending aorta, relative to *Ldlr*^{-/-}; *Gsk3a*^{+/+} mice (Figure 2, A–C). Frozen cross sections of aortic sinus were stained with Oil Red O to identify neutral lipids. Compared with HFD-fed GSK3^{+/+} controls, lesions from GSK3 α -deficient mice contained significantly less neutral lipids (Supplemental Figure S5A). This difference was proportional to the decreased lesion area in these mice.

Consistent with these findings, immunofluorescence staining with antibodies specific for α actin and F4/80 show that the lesions in GSK3 α -deficient mice have proportionally fewer intimal vascular smooth muscle cells and proportionally fewer intimal macrophages/foam cells in the artery wall (Figure 2D). No significant atherosclerotic lesion formation was observed in the descending aortas of any of the mice (Supplemental Figure S5B). Together, these results suggest that GSK3 α deficiency is atheroprotective, and the results are consistent with the possibility that GSK3 α plays a pro-atherogenic role in HFD-induced atherogenesis.

Ldlr/*Gsk3a* Double-Knockout Mice Have Attenuated HFD-Induced Hepatic Steatosis

Hepatic steatosis is a common metabolic condition in several *Apoe*^{-/-} and *Ldlr*^{-/-} mouse models of accelerated atherosclerosis.^{16,25} We have previously shown that valproate supplementation can reduce hepatic steatosis in hyperglycemic and hyperhomocysteinemic *Apoe*^{-/-} mice, suggesting that GSK3 α/β may be involved in the

Figure 4 Effect of Glycogen synthase kinase 3 α deficiency on the unfolded protein response in high fat diet (HFD)-fed *Ldlr*^{-/-} mice. **A:** Quantification of mRNA transcripts encoding GRP78 or CHOP normalized to β -actin from RNA isolated from the livers of chow- or HFD-fed *Ldlr*^{-/-}; *Gsk3α*^{+/+}, *Ldlr*^{-/-}; *Gsk3α*^{+/-}, and *Ldlr*^{-/-}; *Gsk3α*^{-/-} mice, as indicated. **B:** Representative cross sections of aortic sinus from 15-week-old HFD-fed *Ldlr*^{-/-}; *Gsk3α*^{+/+}, *Ldlr*^{-/-}; *Gsk3α*^{+/-}, and *Ldlr*^{-/-}; *Gsk3α*^{-/-} mice stained with an antibody against CHOP. An age-matched HFD-fed *Ldlr*^{-/-}; *Gsk3α*^{+/+} section probed with preimmune rabbit IgG is shown. Immunofluorescence intensity was quantified and is presented as relative intensity/lesion area. **P* < 0.05 relative to chow-fed mice of the same genotype; †*P* < 0.05 relative to *Gsk3α*^{+/+} mice fed the same diet (*n* = 4 per group; **A**); ‡*P* < 0.05 relative to *Gsk3α*^{+/+} mice (**B**). L, atherosclerotic lesions.

development of this condition.²³ To examine the effect of GSK3 α deficiency, hepatic lipids were examined by Oil Red O staining of frozen liver sections and by biochemical assays for total cholesterol and triglycerides. No measurable differences in hepatic lipid content were detected in mice fed a chow diet (Figure 3). HFD induced significant hepatic lipid accumulation in *Ldlr*^{-/-} (Figure 3) and, to a lesser extent, *Ldlr*^{+/-} (Supplemental Figure S6) mice. GSK3 α deficiency resulted in significantly less hepatic lipid accumulation in *Ldlr*^{-/-} and *Ldlr*^{+/-} mice. These results suggest that GSK3 α plays a role in hepatic lipid metabolism. However, GSK3 α deficiency did not significantly affect the plasma lipid profiles in *Ldlr*^{-/-} or *Ldlr*^{+/-} mice (Supplemental Figure S7).

The Effect of GSK3 α Deficiency on the Hepatic and Vascular ER Stress Response

HFD feeding has been previously associated with increased ER stress levels, and we and others have shown that conditions of ER stress and/or induction of the UPR can activate

GSK3 α / β .^{9,14,23,28} Real-time PCR was used to analyze hepatic transcript levels of UPR genes encoding GRP78 and CHOP (Figure 4A). Consistent with previous reports, *Ldlr*^{-/-} mice fed HFD had a significant elevation of both of these ER stress response genes relative to chow-fed controls. In chow-fed mice, GSK3 α deficiency did not significantly alter the expression levels of hepatic transcripts encoding GRP78 or CHOP, compared with chow-fed *Gsk3α*^{+/+} mice. However, in livers of HFD-fed mice, GSK3 α deficiency was associated with an elevation in GRP78 mRNA and a significant decrease in CHOP transcript levels, relative to HFD-fed *Gsk3α*^{+/+} controls.

To investigate CHOP protein levels in atherosclerotic lesions, cross sections of aortic sinus from HFD-fed *Ldlr*^{-/-}; *Gsk3α*^{+/+}, *Ldlr*^{-/-}; *Gsk3α*^{+/-}, and *Ldlr*^{-/-}; *Gsk3α*^{-/-} mice were immunostained with an antibody against CHOP. Immunofluorescence was quantified and normalized to lesion area (Figure 4B). Consistent with observations in the liver, GSK3 α deficiency was associated with a decrease in the UPR protein CHOP. This is the first indication that GSK3 α may directly or indirectly modulate the response to ER stress. No significant GSK3 α -associated changes were

Figure 5 Glycogen synthase kinase 3 α deficiency significantly alters the expression of IL-10 in macrophages. **A:** Cultured peritoneal macrophages from high fat diet (HFD)-fed *Ldlr*^{-/-}; *Gsk3 α* ^{+/+}, *Ldlr*^{-/-}; *Gsk3 α* ^{+/-}, and *Ldlr*^{-/-}; *Gsk3 α* ^{-/-} mice were challenged with 5 mmol/L glucosamine (GlcN) or 4 μ g/mL tunicamycin (Tm) for 18 hours. Total RNA was isolated, and transcript levels of IL-1 β , IL-6, TNF- α , and IL-10 were determined by real-time PCR. **B:** Quantification of IL-10 in plasma from chow- and HFD-fed *Ldlr*^{-/-}; *Gsk3 α* ^{+/+}, *Ldlr*^{-/-}; *Gsk3 α* ^{+/-}, and *Ldlr*^{-/-}; *Gsk3 α* ^{-/-} mice by ELISA. *n* = 3 per group (**A** and **B**). **P* < 0.05 relative to untreated macrophages of the same genotype (**A**); **P* < 0.05 relative to chow mice of the same genotype (**B**); †*P* < 0.05 relative to similarly treated *Gsk3 α* ^{+/+} macrophages (**A**); †*P* < 0.05 relative to *Gsk3 α* ^{+/+} mice fed the same diet (**B**).

observed when lesions were immunostained with an antibody against KDEL, which detects ER-resident proteins, including the UPR chaperones GRP78 and GRP94 (Supplemental Figure S8).

GSK3 α Deficiency Promotes ER Stress–Induced IL-10 Expression

ER stress has been implicated in the activation of proinflammatory pathways.^{8,29} To investigate the effects of GSK3 α deficiency on ER stress–induced inflammation, thioglycolate-elicited peritoneal macrophages were treated *in vitro* with 5 mmol/L glucosamine or 4 μ g/mL tunicamycin, and total cellular RNA was isolated and the activation of the UPR was verified (Supplemental Figure S9A). Transcripts encoding IL-1 β , IL-6, tumor necrosis factor (TNF)- α , and IL-10 were quantified by real-time PCR and normalized to β -actin mRNA (Figure 5A). The transcript levels of IL-1 β and TNF- α were not significantly altered by ER stress and/or GSK3 α deficiency. Exposure to tunicamycin significantly induced IL-6 expression in all macrophage genotypes; however, the

magnitude of this effect was lower in GSK3 α -deficient cells. A fourfold (*P* < 0.05) increase in IL-10 expression was detected in GSK3 α -deficient macrophages.

To determine whether GSK3 α deficiency was associated with alterations in circulating IL-10 levels *in vivo*, we performed an ELISA on plasma isolated from 15-week-old chow- and HFD-fed mice. No significant differences were detected in IL-10 concentrations in any of the chow-fed mice (Figure 5B). GSK3 α deficiency was associated with significantly elevated levels of IL-10 in HFD-fed mice.

To determine whether the observed effects on cytokine expression could be reproduced in a human macrophage cell line, Thp-1–derived macrophages were treated with ER stress–inducing agents, including 2.5 μ g/mL tunicamycin, 5 mmol/L glucosamine, and 600 μ mol/L palmitic acid for 18 hours in the presence or absence of 4 μ mol/L GSK3 α / β inhibitor CT99021. Activation of the UPR was verified (Supplemental Figure S9B). Transcripts encoding IL-1 β , IL-6, IL-8, and IL-10 were quantified by real-time PCR and normalized to β -actin mRNA levels. Exposure to ER stress–inducing agents and/or GSK3 α / β inhibition did not

Figure 6 Endoplasmic reticulum (ER) stress-inducing agents promote IL-10 expression when glycogen synthase kinase (GSK) 3 α / β is inhibited. Thp-1-derived macrophages were challenged for 18 hours with 2.5 μ M tunicamycin (Tm), 5 mmol/L glucosamine (GlcN), or 600 μ M palmitic acid (PA) in the presence or absence of 4 μ M/L GSK3 α / β inhibitor CT99021. Total RNA was isolated and transcript levels for IL-1 β , IL-6, IL-8, and IL-10 were determined by real-time PCR. $n = 3$. * $P < 0.05$ relative to untreated macrophages; † $P < 0.05$ relative to similarly treated *Gsk3 α ^{+/-}* macrophages.

significantly affect IL-1 β , IL-6, or IL-8 mRNA levels (Figure 6). However, GSK3 α / β inhibition was associated with significantly enhanced ER stress-induced IL-10 expression. These results are consistent with the findings in primary GSK3 α -deficient macrophages.

Discussion

Results from our previous studies suggest that signaling through GSK3 α / β may play an important role in the molecular mechanism by which various cardiovascular risk factors, including obesity, dyslipidemia, and hyperglycemia, promote the development of atherosclerosis.^{13,16,23} Specifically, we hypothesize that GSK3 α / β may be a link between ER stress and the activation of pro-atherogenic processes in macrophages, including lipid accumulation and inflammation. The interpretation of these data has been restricted by the lack of specificity of the orally available small-molecule GSK3 α / β inhibitors used in our mouse models, as well as the inability of these systems to discriminate between the roles of GSK3 α and β . In this study, we specifically investigated the role of GSK3 α in HFD-induced atherosclerosis using an *Ldlr*/*Gsk3 α* double-knockout mouse model. Our results show that when placed on a HFD, *Ldlr*^{-/-}/*Gsk3 α* ^{+/-} and *Ldlr*^{-/-}/*Gsk3 α* ^{-/-} mice develop significantly smaller atherosclerotic lesions and have significantly less hepatic lipid accumulation compared with *Ldlr*^{-/-}/*Gsk3 α* ^{+/-} control mice. Studies performed in peritoneal macrophages isolated from the different strains show that under conditions of ER stress, GSK3 α -deficient macrophages express elevated levels of the anti-inflammatory cytokine IL-10, relative to *Gsk3 α* ^{+/-} macrophages. These findings support a specific pro-atherogenic role for GSK3 α . Furthermore, they are consistent with a model in which elevations in ER

stress levels, associated with various cardiovascular risk factors, may signal through GSK3 α to induce the activation of pro-atherogenic pathways.

In contrast to *Gsk3 β* ^{-/-} mice, which die of liver failure and heart defects during midgestation,²⁰ mice lacking GSK3 α are viable and have no overt phenotype.^{21,22} In an outbred genetic background, GSK3 α deficiency was associated with enhanced insulin sensitivity and glucose tolerance.²¹ These metabolic effects appear to vary depending on strain because they were not observed in inbred C57Bl6 *Gsk3 α* ^{-/-} mice.²² These findings suggest that GSK3 α does not play a direct role in glucose homeostasis. Our results, from mice with an inbred C57Bl6 genetic background, are consistent with this report. We observed no differences in blood glucose or oral glucose tolerance, in *Ldlr*^{-/-} mice with 0, 1, or 2 copies of the allele encoding functional GSK3 α . Furthermore, GSK3 α -deficient mice have similar body weights and plasma lipid concentrations compared with age-matched *Ldlr*^{-/-} mice fed the same diet.

Ldlr^{-/-} mice develop hepatic steatosis and advanced atherosclerotic lesions when fed a diet containing relatively high levels of fat and cholesterol. These pathological conditions are associated with elevated levels of hepatic and vascular ER stress.^{24,30} Although previous studies have shown that ER stress-inducing agents can activate inflammatory pathways⁸ and promote triglyceride and cholesterol accumulation^{31,32} in cultured cells, a direct and causative link between ER stress and atherosclerosis has yet to be demonstrated. The strongest data in support of a causative role for ER stress come from experiments performed in the *Ldlr*^{-/-}/*Ddit3*^{-/-} mouse model. These mice have an impaired ability to activate specific components of the UPR and exhibit attenuated HFD-induced atherosclerosis compared with *Ldlr*^{-/-} controls.³⁰ Interestingly, *Ldlr*^{-/-}/*Gsk3 α* ^{-/-} mice express lower levels of CHOP mRNA in

hepatocytes and atherosclerotic lesions than *Ldlr*^{-/-}; *Gsk3 α* ^{+/-} mice. This suggests that GSK3 α plays a role in downstream signaling of the ER stress response pathway. Our findings also suggest that GSK3 α is an important link between ER stress and the regulation of inflammatory responses. Inhibition or elimination of GSK3 α appears to be sufficient to enhance anti-inflammatory cytokine IL-10 production under conditions of ER stress. This could have a significant effect on the development and/or progression of atherosclerosis because overexpression of IL-10 in macrophages has been shown to be sufficient to attenuate atherosclerosis in *Ldlr*^{-/-} mice.³³

Existing evidence supports a role for GSK3 α/β in the modulation of proinflammatory and anti-inflammatory responses; however, the specific mechanisms are not fully understood.³⁴ In monocytes, macrophages, and other cells of the innate immune system, inhibition of GSK3 α/β suppressed Toll-like receptor–induced expression of proinflammatory cytokines, including IL-1 β and IL-6, but enhanced the expression of the anti-inflammatory cytokine, IL-10.³⁵ These effects may, at least in part, be attributed to GSK3 β signaling through STAT3 and STAT5.³⁶ Other studies have suggested that inhibition of GSK3 α/β by the Janus-activating kinase/phosphatidylinositol 3-kinase/AKT pathway relieves GSK3 α/β -mediated inhibition of cAMP-responsive element binding, a transcription factor involved in IL-10 expression.^{37–39} Alternatively, GSK3 α/β may be involved in JNK/AP1 signaling, which has been shown to regulate IL-10.⁴⁰ This is an intriguing possibility because ER stress is known to activate JNK.⁴¹ All of these studies have focused on GSK3 β , or have used small-molecule inhibitors that do not discriminate between GSK3 α and GSK3 β . We present the first study to show a direct effect of GSK3 α deficiency on IL-10 expression. Future *in vivo* and *in vitro* investigations will be directed toward delineating the physiologically relevant mechanisms by which signaling through GSK3 α may promote atherosclerosis.

In this study, IL-1 β and TNF- α expression levels were not significantly affected by either GSK3 α deficiency or exposure to ER stress. Interestingly, IL-6 levels were generally suppressed in macrophages that were deficient for one or both copies of GSK3 α . However, this effect appeared to be independent of ER stress, because GSK3 α deficiency did not impair ER stress–induced IL-6 expression. This finding suggests that ER stress/UPR activation signals through another pathway(s), perhaps involving GSK3 β , to up-regulate IL-6.

The effects of GSK3 α deficiency on the expression of specific cytokines may be indicative of isolated and specific changes in gene programming, or a more profound change in macrophage differentiation that may direct monocytes toward a distinct macrophage subtype. Future studies will be required to further characterize the innate immune system in these different mouse lines and to define the role that GSK3 α plays in the attenuation of HFD-induced accelerated atherosclerosis.

There are several limitations associated with the use of whole body GSK3 α knockout mice in these experiments that

affect the interpretation of the results. Specifically, we cannot discern whether the attenuated atherosclerotic development is a direct result of GSK3 α in cells of the aorta wall, an indirect effect of altered lipid metabolism in the liver, or the result of some unforeseen effect on a different tissue. The lack of any detectable changes in lipid profile supports a mechanism involving local alterations in intimal macrophage/foam cell metabolism. However, GSK3 inhibition has also been shown to improve the functionality of circulating endothelial progenitor cells and enhance arterial repair.⁴² Bone marrow transplantation or tissue-specific GSK3 α and GSK3 β knockout models will have to be established in atherogenic mouse strains to directly distinguish the contribution of vascular versus hepatic GSK3 activity and to determine the specific roles of GSK3 α versus GSK3 β .

To summarize, we present data showing that *Ldlr*^{-/-} *Gsk3 α* ^{+/-} and *Ldlr*^{-/-} *Gsk3 α* ^{-/-} mice are resistant to HFD-induced accelerated atherosclerosis and the development of hepatic steatosis. Furthermore, these results link the pathological conditions to specific ER stress–GSK3 α signaling pathways that regulate cytokine expression. The lack of an overt phenotype associated with the GSK3 α deficiency suggests that specific GSK3 α inhibition may be a viable strategy for the development of novel and effective anti-atherogenic interventions.

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Supplemental Data

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