

HYPERGLYCEMIA-INDUCED ATHEROSCLEROSIS

INVESTIGATION INTO THE ROLE OF THE HEXOSAMINE BIOSYNTHESIS
PATHWAY IN HYPERGLYCEMIA-INDUCED ATHEROSCLEROSIS

By

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ABSTRACT

Diabetes mellitus dramatically increases the risk for atherosclerotic cardiovascular disease. It has been established that chronic hyperglycemia promotes an increase in glucose flux through the hexosamine biosynthesis pathway (HBP). Central to this pathway is glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme controlling the conversion of glucose to glucosamine. We have shown that glucosamine is a potent inducer of endoplasmic reticulum (ER) stress, which is characterized by the accumulation of misfolded proteins in the ER. Chronic ER stress can initiate a multifaceted response that results in lipid accumulation, inflammation and apoptosis: the hallmark features of atherosclerosis. We hypothesized that conditions of chronic hyperglycemia, associated with diabetes mellitus, can accelerate the development of atherosclerosis by a mechanism that involves increased HBP flux resulting in glucosamine-induced ER stress and the subsequent activation of pro-atherogenic pathways. In support of the hypothesis we found that glucosamine-supplemented apoE^{-/-} mice had elevated levels of ER stress and atherosclerosis. Mechanistically, our data showed that glucosamine induced ER stress by interfering with the lipid-linked oligosaccharide biosynthesis pathway and protein N-glycosylation. These findings support a model by which conditions of hyperglycemia promote vascular complications through a glucosamine-intermediate.

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TABLE OF CONTENTS

1. Introduction	1
1.1. Atherosclerosis	1
1.2. Diabetes mellitus	2
1.3. Potential mechanisms linking diabetes to atherosclerosis	4
1.4. The hexosamine pathway	5
1.5. The endoplasmic reticulum and the unfolded protein response	7
1.6. ER stress and atherogenesis	11
1.7. Glucosamine-induced ER stress	11
1.8. Hyperglycemia, ER stress and accelerated atherosclerosis	13
1.9. ER stress in patients with metabolic syndrome	17
1.10. Targets for potential therapeutic intervention	17
1.11. Glutamine:fructose-6-phosphate amidotransferase (GFAT)	21
1.12. Remarks	21
2. Rationale, hypothesis and objectives of study	23
3. Investigation of the effects of glucosamine-supplementation on atherogenesis in	
apoE ^{-/-} mice	26
3.1. Rationale	26
3.2. Hypothesis	26
3.3. Objectives	26
3.4. Abstract	28
3.5. Introduction	29

3.6. Materials and methods	31
3.7. Experimental results	37
3.8. Discussion	58
3.9. References	62
4. Investigation of the effects of glucosamine on lipid-linked oligosaccharide biosynthesis <i>in vitro</i> and <i>in vivo</i>	67
4.1. Rationale	67
4.2. Hypothesis	67
4.3. Objectives	68
4.4. Abstract	69
4.5. Introduction	70
4.6. Methods	72
4.7. Results	75
4.8. Discussion	97
4.9. References	101
5. Examination of the use of thioflavin T as a direct indicator of ER stress in cultured cells and in tissue sections	110
5.1. Rationale	110
5.2. Hypothesis	110
5.3. Objectives	111
5.4. Abstract	113
5.5. Introduction	114

5.6. Methods	116
5.7. Results	119
5.8. Discussion	145
5.9. Conclusions	148
5.10. References	149
6. Discussion	155
7. References (thesis introduction and discussion)	165

LIST OF FIGURES

Introduction Figures

Figure 1. The unfolded protein response to endoplasmic reticulum stress	9
Figure 2. Analysis of aortic root from normoglycemic, STZ-injected hyperglycemic, and STZ-injected insulin treated apoE ^{-/-} mice	15
Figure 3. Working model of diabetes associated accelerated atherothrombosis	19

Chapter #1 Figures: Glucosamine-supplementation in apoE^{-/-} mice

Figure 1. Identifying changes in intracellular glucosamine levels	46
Figure 2. Activation of the ER stress response	48
Figure 3. Measuring hepatic lipid concentration	52
Figure 4. Analysis of atherosclerotic lesion development	54
Figure 5. Effect of valproate on atherosclerotic lesion size	56
Supplemental Figure 1. Control stains for the immunohistochemical procedure	35
Supplemental Figure 2. The effect of glucosamine supplement on the plasma lipid profile	41
Supplemental Figure 3. The effect of an oral glucose or glucosamine challenge on blood glucose levels	43

Chapter #2 Figures: Glucosamine disrupts LLO biosynthesis

Figure 1. Elevated concentrations of glucosamine disrupt protein glycosylation	76
Figure 2. Glucosamine disrupts lipid-linked oligosaccharide biosynthesis <i>in vitro</i>	80

Figure 3. Glucosamine disrupts LLO biosynthesis and induces ER stress in a time and concentration dependent manner in MEF cells	82
Figure 4. DTT and thapsigargin induce ER stress but do not disrupt LLO biosynthesis	87
Figure 5. 4-Phenylbutyric acid mitigates glucosamine-induced ER stress but not LLO disruptions	89
Figure 6. Glucosamine induce ER stress and atherogenesis in apoE ^{-/-} mice	93
Figure 7. Glucosamine-supplementation disrupts LLO biosynthesis in apoE ^{-/-} mice	95
Chapter #3 Figures: Detection of ER stress with thioflavin T	
Figure 1. Increased ThT fluorescence in the presence of thapsigargin	121
Figure 2. ThT fluorescence intensity corresponds to ER stress-induced activation of the unfolded protein response	123
Figure 3. Exposure to the chemical chaperone, 4PBA, reduces UPR activation and ThT fluorescence	126
Figure 4. Co-localization of ThT and KDEL in the ER of thapsigargin-treated MEFs	132
Figure 5. Exposure to different ER stress-inducing agents enhances ThT fluorescence	134
Figure 6. Enhanced ThT fluorescence is detectable within 60 minutes	136
Figure 7. ER stress promotes enhanced ThT fluorescence in different cell types	138
Figure 8. Enhanced ThT fluorescence is detectible in mouse models of hepatic ER stress	141
Supplemental Figure 1. Exposure to the chemical chaperone, TUDCA, reduces ThT fluorescence	128

Supplemental Figure 2. Additional controls for the ThT and KDEL staining in cell culture and mouse tissue	143
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Discussion

Figure 1. Summary our working model of hyperglycemia-induced accelerated atherosclerosis	157
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LIST OF TABLES

Supplemental Table 1. The general effects of glucosamine- and mannitol- supplementation, and STZ-induced hyperglycemia in 15 week old female apoE-/- mice	39
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LIST OF ABBREVIATIONS

AGE	advanced glycation end-product
ANOVA	analysis of variance
ApoE-/-	apolipoprotein E-deficient
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
CHOP	C/EBP homologous protein
CVD	cardiovascular disease
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
eIF2 α	eukaryotic initiation factor 2 alpha
ER	endoplasmic reticulum
E-selectin	endothelial-leukocyte adhesion molecule-1
FACE	fluorescent-assisted carbohydrate electrophoresis
FBS	fetal bovine serum
G6Pase	glucose-6-phosphatase

GADD153	growth arrest and DNA damage 153
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAT	glutamine-fructose-6-phosphate amidotransferase
GlcN	glucosamine
GlcNAc	N-acetyl glucosamine
GLUT	glucose transporter
GRP78/BiP	glucose regulated protein 78
GSK3	glycogen synthase kinase 3
HDAC	histone deacetylase
HDL	high density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	human hepatocarcinoma
HG	hyperglycemic or hyperglycemia
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMG-CoAR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
KDEL	lysine-aspartic acid-glutamic acid-leucine

LDL	low density lipoprotein
LLO	lipid-linked oligosaccharide
MEF	mouse embryonic fibroblast
mLDL	modified low density lipoprotein
MMP	matrix metalloproteinase
NAD ⁺ or NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF- κ B	nuclear factor-kappaB
NG	normoglycemic or normoglycemia
O-GlcNAc	O-linked N-acetylglucosamine
OGT	O-GlcNAc transferase
OGTT	oral glucose tolerance test
oxLDL	oxidized low density lipoprotein
PAI-1	plasminogen activator inhibitor-1
4-PBA	4-phenylbutyric acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PERK	ER-resident PKR-ER-related kinase
PI3K	phosphatidylinositol 3-kinase
PKB/Akt	protein kinase B/Akt
PKC	protein kinase C

PS	penicillin/streptomycin
PUGNAc	O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate
qPCR	quantitative polymerase chain reaction
RAGE	receptor for advanced glycation endproduct
RNA	ribonucleic acid
ROS	reactive oxygen species
s.d.	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s.e.	standard error
Ser	serine
VSMC	vascular smooth muscle cell
SP1	specificity protein 1
SR-A	scavenger receptor-A
SREBP	sterol regulatory element binding protein
STZ	streptozotocin
T1DM	type I diabetes mellitus
T2DM	type II diabetes mellitus
TBST	tris-buffered saline tween-20
Thr	threonine
ThT	Thioflavin T

T _m	tunicamycin
TNF- α	tumor necrosis factor-alpha
TUDCA	taurine-conjugated ursodeoxycholic acid
Tyr	tyrosine
UDP-GlcNAc	uridine diphosphate-N-acetylglucosamine
UPR	unfolded protein response
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoprotein
VPA	valproic acid
WT	wild-type

1 INTRODUCTION

1.1. Atherosclerosis

Atherosclerosis is an inflammatory disease characterized by the gradual thickening of large artery walls due to the accumulation of atheromatous deposits [1]. This disease is the major underlying cause of cerebro- and cardiovascular disease (CVD). Atherosclerotic lesions typically develop at sites of endothelial dysfunction, and are localized in regions of the artery where linear blood flow is disrupted (ie. bifurcations) [2]. Conditions associated with endothelial dysfunction include hyperglycemia, insulin resistance, hypercholesterolemia, hypertension and obesity. Upon damage or dysfunction, endothelial cells (EC) increase the expression of monocyte and T-cell directed adherence molecules. These adherent molecules include the P, E, and L selectin, intracellular adhesion molecule (ICAM), and the vascular cell adhesion molecule (VCAM); which promote the binding of leukocytes to the EC surface [3]. Monocyte and T-cell recruitment into the intimal layer is then facilitated by factors released by ECs, including monocyte chemoattractant protein 1 (MCP-1) and matrix metalloproteinase 9 (MMP-9) [4, 5]. Intimal monocytes differentiate into macrophages that take up LDL and modified-LDL particles that have entered the intima. If the LDL content is high, macrophages become engorged (foam cells) and further amplify the inflammatory response to vascular injury by secreting cytokines including TNF- α and IL-1 β [1, 3]. The accumulation of foam cells in the intima of the artery wall forms a “fatty streak”. For unknown reasons, some fatty streaks continue to grow and evolve into advanced lesions. Advanced lesions are characterized by the cytokine-induced migration of vascular smooth muscle cells

(VSMC) into the intima. VSMC synthesize and secrete extracellular matrix proteins that collectively form a protective fibrous cap over the lesion. Ultimately, lipid engorged foam cells can undergo apoptosis and create a region known as the necrotic core: a key feature in plaque instability. Other factors contributing to plaque destabilization are the macrophage/foam cell secreted collagenases (ie. MMP-1) that breakdown the protective fibrous cap [6]. Together, these effects disrupt the stability of the plaque and leave it predisposed to rupture.

When lesion rupture occurs blood comes into contact with cellular debris, including lipids and tissue factor, in the necrotic core initiating platelet adherence and thrombosis [7]. Depending upon its size, the thrombus may occlude the artery blood flow and cause a myocardial infarction, or rupture and cause a stroke.

1.2. Diabetes mellitus

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by insufficient insulin action leading to chronic hyperglycemia. Type 1 diabetes (T1D) develops from a lack of insulin production due to the autoimmune-initiated destruction of pancreatic β cells. The onset of type 2 diabetes (T2D) occurs from an insufficient insulin effect that develops in combination with insulin resistance and progressive β cell dysfunction.

Over the last few decades, there has been a dramatic, worldwide increase in the incidence of diabetes mellitus. Driven by changes in lifestyle and an escalating rate of obesity, the number of individuals with diabetes may already be as high as 350 million

[8, 9]. Diabetes mellitus is a major, independent risk factor for cardiovascular disease (CVD) and individuals with diabetes are 2 to 3 times more likely to die from CV causes than people with no history of diabetes, even after controlling for other CV risk factors [10-12]. These individuals are also at increased risk of diseases that are associated with CVD and atherosclerosis including hypertension and renal failure. The increasing incidence of diabetes means that the burden of this chronic disease on health care resources will continue to rise for the foreseeable future.

It is not clear why individuals with diabetes are predisposed to CVD. Although hyperglycemia is recognized as an independent risk factor for myocardial infarction and stroke [13, 14], recent reports from clinical trials examining the effects of intensive blood glucose lowering on CV risk, including ACCORD (Action to Control Cardiovascular Risk in Diabetes) [15], ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation) [16], UKPDS (United Kingdom Prospective Diabetes Study) [17] and VADT (Veterans Affairs Diabetes Trial) [18], suggest that the relationship between hyperglycemia and CVD is complex. Despite a vast amount of research, currently available treatments show only limited CV benefit and CVD continues to be the major cause of mortality. A better understanding of the molecular link between diabetes and CVD is required in order to facilitate the development of specific and more effective interventions.

1.3. Potential mechanisms linking diabetes to atherosclerosis

Several mechanisms have been proposed to explain the pro-atherogenic effects of diabetes and hyperglycemia. In general these have focused upon the intracellular effects of elevated levels of glucose, and the increased availability of glucose metabolites, in cells of the vascular wall. There is evidence that hyperglycemia is associated with increased aldose reductase activity that can lead to increased consumption of NADPH and depletion of GSH levels resulting in elevated levels of reactive oxygen species (ROS) and subsequent cellular damage [19, 20]. Glucose-induced PKC activation has been implicated in decreased endothelial vasodilation [21] and increased production of ROS [22] that could contribute to endothelial dysfunction. It has also been proposed that the conversion of sorbitol to 3-deoxyglucosone can feed into the production of advanced glycation endproducts (AGEs). AGEs are formed through a nonenzymatic process, known as the Maillard reaction, involving the reaction of the aldehyde groups of reducing sugars with the amino groups of proteins [23, 24]. There are several potential ways that AGE-modified proteins could be damaging; the formation of AGEs may alter protein function [25], disrupt the extracellular matrix [25, 26] and/or lead to the modification of lipoprotein particles thereby increasing their atherogenicity. However it now appears that the predominant vascular effect of AGEs occurs through their interaction with RAGE (receptor for AGE) found on macrophages, endothelial and smooth muscle cells [27-29]. The AGE-RAGE interaction triggers a signal transduction cascade that results in the production of intracellular ROS that can initiate an inflammatory response [30].

Each of the above responses is associated with increased oxidative stress and activation of pro-inflammatory pathways in vascular cells [31]. While pre-clinical evidence supports a causative role for oxidative stress in atherogenesis [32-35], virtually every well-controlled clinical trial has failed to show a cardiovascular benefit in diabetic patients receiving anti-oxidant supplements [36-40]. There are several ways to rationalize this apparent paradox by questioning; the specific anti-oxidants tested, the doses prescribed and/or the power and duration of the trials themselves. However, these clinical observations may be indicative of the existence of other important molecular mechanisms or pathways that play a causative role in diabetic atherogenesis in addition to oxidative stress.

1.4. The hexosamine pathway

Conditions of hyperglycemia also result in the shunting of excess intracellular glucose through the hexosamine biosynthetic pathway (HBP). In a typical cell, under normo-glycemic conditions, 1 to 3% of total glucose will be converted to glucosamine-6 phosphate by the enzyme glutamine:fructose-6 phosphate amidotransferase (GFAT) [41]. When intracellular glucose levels rise, flux through this pathway increases. Furthermore, increased GFAT expression and activity have been reported in tissues from humans with diabetes [42]. The net result is an elevated intracellular concentration of glucosamine. This effect has been observed in cultured cells challenged with elevated concentrations of glucose as well as in vascular and hepatic tissues of hyperglycemic animals [43-46].

Increased hexosamine pathway flux has been implicated in several diabetes-associated complications including; insulin resistance [41, 47], pancreatic β cell death [48], as well as atherosclerosis [49]. The molecule mechanisms that underlie the pathogenic effects of increased HBP flux are not fully understood. Most research has focused upon the role of UDP-N-acetyl-glucosamine (UDP-GlcNAc), the end-product of the HBP pathway and a substrate for both O- and N-linked protein glycation as a causative agent. It is well established that elevated glucosamine concentrations drive the O-linked glycosylation of proteins including transcription factors [50], nuclear pore proteins [51], as well as signaling factors [52] which potentially alters their function, stability and/or activity. Glucosamine has been shown to desensitize insulin-stimulated glucose uptake in both adipocytes [41] and skeletal muscle cells [53], probably by inhibiting the translocation of the glucose transporter, GLUT4, to the cell surface [54]. In addition, increased hexosamine pathway activity can promote the transcription of pro-inflammatory and pro-thrombotic factors including TGF- α , TGF- β and PAI-1 [55-57]. Therefore, the hyperglycemia-induced O-GlcNAc modification of proteins may change gene expression patterns and alter the function of specific factors that contribute to a pro-atherogenic, and pro-thrombotic vascular environment. More studies are required to test this theory and to precisely determine the factors and downstream pathways that may be involved in the acceleration of vascular disease.

UDP-GlcNAc is also a substrate for N-linked protein glycation that occurs in the endoplasmic reticulum (ER). N-glycation is an important post-translational modification of nascent proteins that is critical for proper protein folding [58]. Disruptions in the N-

glycation process can lead to an accumulation of unfolded/misfolded proteins that ultimately disrupt the ER homeostatic balance; this is known as “ER stress”.

1.5. The endoplasmic reticulum and the unfolded protein response

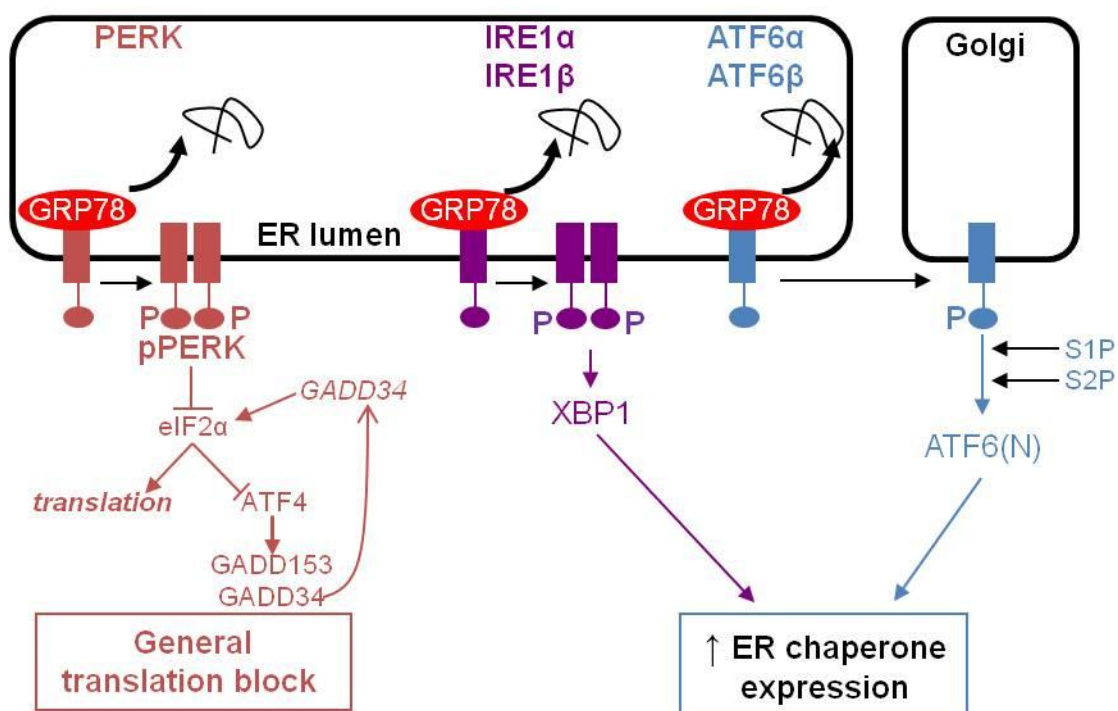
In a typical eukaryotic cell, the ER is responsible for the proper modifying, folding, and trafficking of approximately one third of all proteins. ER localized modifications of nascent proteins include disulfide bond formation and N-linked glycosylation, which are critical to protein folding [58]. Unfolded/misfolded proteins are directed to undergo ER associated degradation (ERAD) and, under physiological conditions, the ER is able to maintain a homeostatic balance between folded and misfolded proteins [59]. When the ER processing capacity is overwhelmed, unfolded/misfolded proteins accumulate and disrupt the ER homeostatic balance; this is known as ER stress.

Traditional ER stress-inducing agents are known to disrupt protein folding by interfering with disulphide bond formation (dithiothreitol) [60], ER Ca^{2+} balance (A23187, thapsagargin) [61], ER membrane structure (palmitate, unesterified cholesterol) [62, 63] or by blocking protein N-glycosylation (tunicamycin) [64]. Conditions of ER stress activate the unfolded protein response (UPR) which functions to restore ER homeostasis (Figure 1). The UPR is a three pronged signaling cascade that is initiated by trans-membrane ER proteins known as Inositol-Requiring Enzyme (IRE)-1, Activating Transcription Factor (ATF)-6, and PKR-like ER Kinase (PERK) [65]. Initiation of these pathways alleviates ER stress by decreasing protein synthesis, increasing ER chaperone

levels, and facilitating degradation of irreversibly misfolded proteins. Under conditions of chronic ER stress, upregulation of pathways involved in lipid accumulation (SREBP) and inflammation (NF- κ B) can occur [43, 66-68]. If the UPR is unable to restore ER homeostasis, pro-apoptotic signaling factors (ie. GADD153/CHOP) are upregulated to initiate programmed cell death [69].

Figure 1. The unfolded protein response to endoplasmic reticulum stress.

ER stress occurs when the capacity of the ER to process/fold proteins is exceeded by the load of nascent proteins entering the ER. The function of the UPR is to re-establish ER homeostasis by decreasing protein flux into the ER (translation block) while increasing the folding capacity of the ER (increased chaperone expression). Conditions of ER stress lead to the dissociation of ER chaperone GRP78 from the trans-ER-membrane signaling factors PERK, IRE1 and ATF6, resulting in their activation. Activated PERK phosphorylates and inhibits the activity of eIF2 α – an essential factor in general protein translation. PERK is also involved with the downstream activation of transcription factors including ATF4 and GADD153/CHOP. Activated IRE1 assists in the alternative splicing of XBP-1 resulting in the translation of a transcription factor, XBP-1, which is involved in upregulation of the expression of ER chaperones. Activated ATF6 translocates to the Golgi where proteases S1P and S2P release an N-terminal transcription activation domain that works in concert with XBP-1 to upregulate ER chaperone expression.



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1.6. ER stress and atherogenesis

There is increasing experimental evidence in support of a direct and causative role for ER stress in the development and/or progression of atherosclerosis. First, several independent risk factors for CVD, including hyperglycemia [43], hyperhomocysteinemia [12, 68], obesity [70] and elevated levels of palmitate [71] and unesterified cholesterol [72], have been associated with ER stress, suggesting that this pathway may represent a common or unifying mechanism of accelerated atherogenesis [73, 74]. Secondly, activation of the UPR has been noted at all stages of atherosclerotic development, from a fatty streak to an advanced occlusive plaque [75]. Third, conditions of ER stress can activate/dysregulate metabolic pathways that are directly involved in the development of atherosclerotic lesions. ER stress-inducing agents promote lipid accumulation by activating the sterol regulatory element binding proteins (SREBPs), which are transcription factors that control lipid biosynthesis and uptake [68, 76, 77]. ER stress-inducing agents also activate NF- κ B, the transcription factor responsible for promoting inflammatory gene expression [78, 79]. Finally, ER stress has been shown to activate caspases and promote apoptosis of human aortic endothelial cells and other cell types [80, 81]. Together, lipid accumulation, inflammation and endothelial apoptosis are the hallmark features of atherosclerosis [3, 82].

1.7. Glucosamine-induced ER stress

Our lab has recently over-expressed the HBP rate limiting enzyme, GFAT, using an adenoviral expression system in cell culture and measured a significant increase in

UPR gene expression and downstream effects of ER stress including, lipid accumulation, inflammatory gene expression, and apoptotic signaling under hyperglycemic conditions [66]. We have shown that addition of exogenous glucosamine, or increased endogenous production of glucosamine, can disrupt the capacity of the ER to process nascent proteins and initiate an ER stress response. Furthermore, this effect has been observed in cell types that are relevant to the development of atherosclerosis, including human aortic smooth muscle cells, monocyte-derived macrophages and HepG2 cells [43, 44, 80, 81]. Thus, elevated levels of glucosamine may play an important role in ER and cellular dysfunction associated with atherogenesis.

It is not known how increased concentrations of glucosamine (but not mannose) disrupt protein folding in the ER. UDP-N-acetylglucosamine is an essential substrate for both O- and N-linked protein glycosylation and protein glycosylation is an important step in the proper folding of many proteins [58]. It is known that elevated concentrations of glucosamine increase levels of O-linked protein glycosylation [43] and alter N-linked glycosylation patterns of specific proteins including ApoB100 [83]. It is possible that either of these effects could promote ER stress. In cultured HepG2 cells, our lab has shown that PUGNAc, an inhibitor of O-GlcNAcase, increases protein-O-GlcNAc levels but does not promote ER stress [43]. This may suggest that glucosamine-induced ER stress is caused by free and not protein O-linked glucosamine. We hypothesize that increased levels of glucosamine, or a derivative of glucosamine, may interfere with a step in the N-linked glycation of proteins resulting in the production of misfolded proteins and the activation of the UPR.

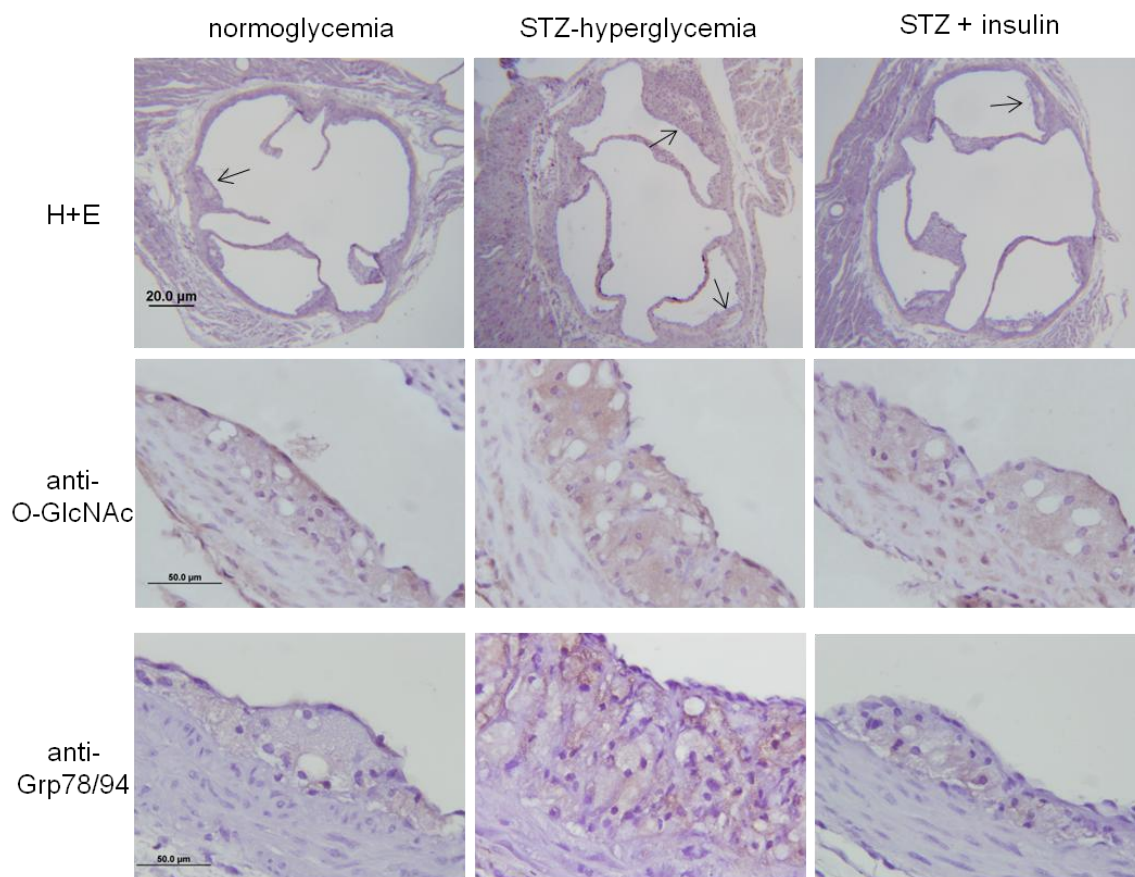
Elevated levels of glucosamine and glucosamine-induced ER stress have been previously implicated in acquired insulin resistance [41, 47, 84, 85]; although, there is some controversy to whether this effect is physiologically relevant in humans. Incubation of relatively high concentrations of glucosamine (1-10 mmol/L) in adipose, muscle or endothelial cell cultures has been implicated in impaired insulin action [85-88]. Furthermore, high levels of intravenously injected glucosamine (plasma concentrations of 0.5-1.8 mmol/L) in both animals and humans have also been shown to cause insulin resistance [89, 90]. The recommended daily dose of oral glucosamine supplements, commonly taken to treat joint pain, are far lower (plasma concentrations of ~3 μ mol/L) and data suggest that these supplements have no effect on insulin sensitivity [91, 92]. Additional studies will be required to determine the effects of chronic hyperglycemia on endogenous, intracellular levels of glucosamine and possible effects on insulin resistance.

1.8. Hyperglycemia, ER stress and accelerated atherosclerosis

To investigate the molecular mechanisms that link hyperglycemia to atherosclerosis we have established a model in which we inject apoE^{-/-} mice with multiple low doses of streptozotocin (STZ) [43, 44, 93]. Using this model we have observed a correlation between hyperglycemia, the accumulation of glucosamine in the artery wall, vascular ER stress and accelerated atherogenesis [43] (Figure 2). Significantly, ER stress levels in the endothelium of hyperglycemic mice increase prior to the development of the atherosclerotic lesions, a result that is consistent with ER stress playing a causative role in lesion development [93]. In addition, accelerated lesion

development is observed in these diabetic mice before the onset of dyslipidemia, suggesting that hyperglycemia is sufficient to independently promote the activation of pro-atherogenic processes [43].

Figure 2. Analysis of aortic root from normoglycemic, STZ-injected hyperglycemic, and STZ-injected insulin treated apoE^{-/-} mice. Hyperglycemic mice show increased vascular O-linked GlcNAc, elevated levels of ER stress markers (GRP78/94) and significantly accelerated atherosclerotic lesion development, relative to normoglycemic controls. Normalization of glucose levels with insulin attenuates O-GlcNAc accumulation, ER stress and atherogenesis.



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1.9. ER stress in patients with metabolic syndrome

There is ample evidence *in vitro* and in animal models to support a role for ER stress in the development and complications of diabetes. Recently, small clinically relevant studies involving humans with metabolic syndrome have been carried out. Patients with diabetic nephropathy have been shown to have increased GFAT expression in glomerular epithelial and mesangial cells, and that GFAT is expressed in most tissues involved in diabetic complications [42, 94]. Isolated pancreatic cells from type 2 diabetics have been shown to have marked expression of ER stress markers [95], increased susceptibility to ER stress compared to non-diabetic controls [96], and that ER stress may contribute to IL-1 β production, mild islet inflammation [97] and β -cell failure [95]. Our lab has recently shown that isolated leukocytes from human subjects with metabolic syndrome, compared to healthy subjects, have elevated levels of ER stress markers, and that there is an association between acute and chronic dysglycemia and ER stress in humans [98]. Each of these trials are consistent with diabetes-associated ER stress playing a clinically relevant role in the pathogenesis of diabetic complications.

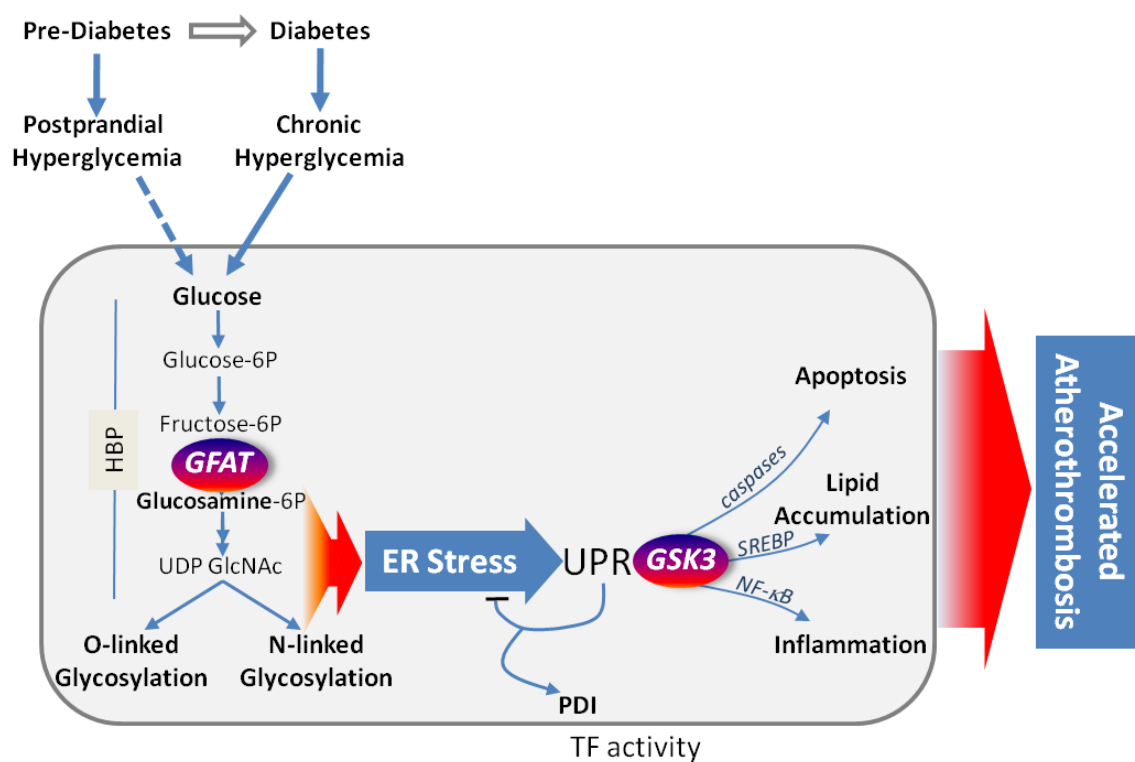
1.10. Targets for potential therapeutic intervention

The identification of a role for ER stress and/or the UPR in the development and progression of diabetes-associated atherosclerosis is significant, not only because it gives us insight on an important disease process, but also because it illuminates novel potential targets for therapeutic intervention (Figure 3). Efforts to develop strategies to manipulate the UPR have already begun, especially with respect to other diseases and disorders in

which ER stress is thought to play a role. At least three general approaches have been used to address this problem. The first involves reducing the levels of ER stress directly by relieving the load of misfolded proteins through the addition of chemical chaperones such as 4-phenylbutyric acid (4-PBA), taurine-conjugated ursodeoxycholic acid (TUDCA) or dimethyl sulfoxide (DMSO) [99-101]. The mechanisms by which these small molecules function to reduce ER stress levels are not well defined. However, such strategies have been shown to be effective *in vitro* and *in vivo*, and 4-phenylbutyric acid has been shown to attenuate atherosclerosis in an apoE^{-/-} mouse model [102]. A second strategy is to augment the protective aspects of the endogenous UPR. This has previously been accomplished through the over-expression of ER resident protein chaperones including GRP78. The third approach is to target some of the detrimental downstream effects of ER stress. Examples of this strategy include the use of salubrinal which inhibits the phosphatase GADD34 from re-activating eIF2 α , thereby maintaining the PERK pathway-induced translation block (Figure 1). Other possible targets for intervention would include pro-inflammatory and/or pro-apoptotic factors such as ASK1, p38MAPK or GADD153/CHOP. Indeed, GADD153/CHOP-deficient mice are resistant to accelerated atherosclerosis [103, 104]. Recently we have identified glutamine:fructose-6-phosphate amidotransferase (GFAT) as an enzyme involved in ER stress and a potential target for therapeutic intervention.

Figure 3. Working model of diabetes associated accelerated atherothrombosis.

Chronic hyperglycemia leads to increased flux through the hexosamine biosynthesis pathway (HBP) resulting in accumulation of UDP-N-acetylglucosamine (UDP-GlcNAc), a substrate for both O- and N-linked protein glycosylation as well as increased levels of ER stress. Disruptions in ER homeostasis lead to activation of the unfolded protein response (UPR) and downstream effects including activation of glycogen synthase kinase (GSK)-3. Our results suggest that ER stress-induced GSK-3 induces pro-atherogenic processes leading to the accelerated development of atherothrombosis.



Beriault, D. and Werstuck, G. Experimental Diabetes Research 2012

1.11. Glutamine:fructose-6-phosphate amidotransferase (GFAT)

The potential role of glucosamine-induced ER stress in diabetic atherogenesis highlights the importance of glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate limiting enzyme in the conversion of glucose to glucosamine, also known as the hexosamine pathway [105, 106]. A central role for GFAT activity in the ER stress pathway is supported by our finding that inhibition of GFAT attenuates glucose-induced ER stress [43] and that overexpression of GFAT is sufficient to promote ER stress in HepG2 cells cultured in normo-glycemic conditions [66]. We are currently developing strategies to modulate GFAT activity *in vitro* and in our mouse models. These tools will be used to investigate the potential effects of regulating GFAT activity on the UPR and on activation of pro-atherogenic processes.

1.12. Remarks

Because of the cardiovascular risks of diabetes and the increasing prevalence of T2D, it is essential that we further our knowledge of how and why diabetes mellitus and hyperglycemia promote cardiovascular disease. Currently, and for the near future, the primary strategy for managing cardiovascular disease in the diabetic population will be through the control of hyperglycemia and through the treatment of associated complications such as hypertension and dyslipidemia using established medications such as ACE inhibitors, statins and fibrates.

The continued identification and investigation of pathways linking hyperglycemia and diabetes mellitus to atherosclerosis is important to the development of new and effective anti-atherosclerotic therapies that are tailored to individuals with diabetes. A great deal of research has been focused upon the role of hyperglycemia in the development and progression of atherosclerosis in cell culture and animal model systems. Several mechanisms have been identified that appear to link glucose to pro-atherogenic processes. The most promising of these; the polyol pathway, PKC activation, the hexosamine pathway and the AGE-RAGE interaction show potential and are actively being evaluated as targets for putative anti-atherogenic therapies. Thus far, however, all interventions targeting the effects of hyperglycemia, including direct glucose lowering, appear to show greater effect in the treatment of microvascular complications than in the control of macrovascular disease. This is likely due, at least in part, to the complexities of atherosclerosis and current limitations of the animal models available to researchers who study the development and progression of atherosclerosis. Additional studies are obviously required to further our understanding of this important disease.

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Beriault, DR. and Werstuck GH. The role of the glucosamine-induced ER stress in diabetic atherogenesis. *Experimental Diabetes Research* 2012; 2012:ID 187018.

2 RATIONALE, HYPOTHESIS AND OBJECTIVES OF STUDY

2.1 Central Hypothesis

We hypothesize that conditions of chronic hyperglycemia, associated with diabetes mellitus, accelerate the development of atherosclerosis by a mechanism that involves increased HBP flux resulting in glucosamine-induced ER stress and the subsequent activation of pro-atherogenic pathways.

2.2 Chapter #1

Investigation of the effects of glucosamine-supplementation on atherogenesis in apoE^{-/-} mice.

2.2.1 Hypothesis

We hypothesize that glucosamine-supplementation of apoE^{-/-} mice will directly induce ER stress and accelerate atherogenesis comparable to what we observe in a diabetic/hyperglycemic mouse model.

2.2.2 Objectives

- 1 Determine the effect of glucosamine-supplementation on atherosclerotic lesion volume in a pro-atherogenic mouse model.
- 2 Determine the effects of glucosamine-supplementation on ER stress levels, plasma lipid profiles and neutral lipids *in vivo*.
- 3 Investigate whether valproate can attenuate glucosamine-induced atherogenesis.

2.3 Chapter #2

Investigation of the effects of glucosamine on lipid-linked oligosaccharide biosynthesis *in vitro* and *in vivo*.

2.3.1 Hypothesis

Elevated concentrations of glucosamine promote ER stress by interfering with lipid-linked oligosaccharide (LLO) biosynthesis, and thereby disrupting N-linked glycosylation and protein folding.

2.3.2 Objectives

- 1 Examine the effects of glucosamine on LLO biosynthesis *in vitro* and *in vivo* using fluorescence-assisted carbohydrate electrophoresis (FACE).
- 2 Examine the relationship between disruptions in LLO biosynthesis and the up-regulation of ER stress markers.
- 3 Conduct western blots to determine the effects of glucosamine on protein levels/N-glycosylation.

2.4 Chapter #3

Examination of the use of thioflavin T as a direct indicator of ER stress in cultured cells and in tissue sections.

2.4.1 Hypothesis

We hypothesize that the fluorophore, thioflavin T, can be utilized as an indicator of ER stress because of its ability to bind to unfolded or aggregated proteins and emit enhanced fluorescence intensity.

2.4.2 Objectives

- 1 Test the ability of thioflavin T to detect ER stress in live cells using known ER stress agents using fluorescent microscopy,.
- 2 Determine if thioflavin T co-localizes with misfolded proteins in the ER using confocal microscopy.
- 3 Determine if thioflavin T can also detect ER stress in fresh frozen tissues from various mouse models.

3 INVESTIGATION OF THE EFFECTS OF GLUCOSAMINE-SUPPLEMENTATION ON ATHEROGENESIS IN APOE^{-/-} MICE

3.1 Rationale

The mechanisms linking diabetes mellitus to accelerated atherosclerosis are unclear. Our lab has previously shown that markers of endoplasmic reticulum (ER) stress are elevated, both *in vitro* and *in vivo*, by conditions of hyperglycemia [43, 44]. Furthermore, ER stress can induce inflammation, lipid accumulation and apoptosis, which are hallmark features of atherosclerosis [68, 80, 81]. Recently we have found that exposure of cultured cells to high concentrations of glucose can induce ER stress by increasing flux through the hexosamine biosynthesis pathway (HBP), which converts the glycolysis metabolite: fructose-6-phosphate, to glucosamine-6-phosphate. The goal of this aim is to better understand the role of glucosamine in diabetes-induced atherosclerosis *in vivo*.

3.2 Hypothesis

We hypothesize that glucosamine-supplementation of apoE^{-/-} mice will directly induce ER stress and accelerate atherogenesis comparable to what we observe in a diabetic/hyperglycemic mouse model.

3.3 Objectives

- 1 Determine the effect of glucosamine-supplementation on atherosclerotic lesion volume in a pro-atherogenic mouse model.
- 2 Determine the effects of glucosamine-supplementation on ER stress levels, plasma lipid profiles and neutral lipids *in vivo*.
- 3 Investigate whether valproate can attenuate glucosamine-induced atherogenesis.

**Glucosamine-supplementation promotes endoplasmic reticulum stress,
hepatic steatosis and accelerated atherogenesis in apoE^{-/-} mice**

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This paper consists of 5 figures, 3 supplemental figures and 1 supplemental table.

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Abstract

Objectives: To determine the effects of glucosamine-supplementation on endoplasmic reticulum (ER) stress levels and atherogenesis, and to investigate the potential role of glucosamine in hyperglycemia-associated accelerated atherosclerosis.

Methods: Five week old apolipoprotein E-deficient (apoE^{-/-}) mice were provided with normal drinking water or water supplemented with 5% glucosamine (w/v) or 5% mannitol (w/v). To induce hyperglycemia, a separate group of apoE^{-/-} mice received multiple low dose injections of streptozotocin (STZ). All mice were provided with a standard chow diet and were euthanized at 15 weeks of age. Hepatic and vascular ER stress levels and atherosclerotic lesion area at the aortic root were determined.

Results: STZ-induced hyperglycemic and glucosamine-supplemented mice had significantly larger and more advanced atherosclerotic lesions than control mice. Indications of ER stress were increased in the livers and atherosclerotic lesions of hyperglycemic and glucosamine-supplemented mice but not in the controls. In glucosamine-supplemented mice accelerated atherosclerosis was independent of detectable changes in blood glucose concentration, glucose tolerance, plasma insulin, or plasma lipid levels.

Conclusion: Similar to hyperglycemia, glucosamine-supplementation promotes ER stress, hepatic steatosis and accelerated atherosclerosis. These findings support a model by which hyperglycemia promotes hepatic and vascular complications via a glucosamine intermediate.

Keywords: atherosclerosis, hepatic steatosis, diabetes mellitus, hyperglycemia, glucosamine, ER stress

1. Introduction

Diabetes mellitus is a major, independent risk factor for cerebro- and cardiovascular disease (CVD) and CVD accounts for approximately 70% of all diabetic mortalities [1]. The molecular mechanisms that link diabetes to the development and progression of CVD are not fully understood. Data from basic studies using vascular cell culture and animal model systems strongly support a role for hyperglycemia in the activation of pro-atherogenic pathways [2, 3]. Epidemiological evidence suggests there is an association between glycemic control and CVD, and hyperglycemia has been identified as an independent risk factor for CVD [4]; however, the specific and causal role of glucose in the accelerated development of atherosclerosis remains controversial.

The unfolded protein response (UPR) is a multifaceted cellular self-defense system that is activated when the capacity of the endoplasmic reticulum (ER) to fold and process nascent proteins is overwhelmed – a condition known as ER stress. Recent evidence from several independent studies suggests that disruptions in ER homeostasis, and/or activation of the UPR is associated with the development of atherosclerosis. Indications of ER stress can be detected in endothelial cells prior to plaque formation [3, 5], as well as throughout the development and growth of the atherogenic plaque [6]. Cardiovascular risk factors including obesity, hyperhomocysteinemia, hypercholesterolemia, and diabetes mellitus have been associated with activation of the UPR *in vivo* [7-9] and elevated intracellular concentrations of unesterified cholesterol, palmitate, homocysteine and glucose have been shown to induce ER stress in a number of cultured cell systems [10-13]. The mechanisms by which these agents induce ER stress

and the links between activation of the UPR and induction of downstream, pro-atherogenic processes are not fully understood. However, it is now apparent that ER stress-inducing agents can promote cellular responses including dysregulated lipid biosynthesis [13, 14], activation of inflammatory cascades [15, 16] and induction of apoptosis [11, 12] that may be directly involved in atherogenesis.

In cultured skeletal muscle and aortic endothelial cells, elevated concentrations of glucose have been shown to induce ER stress by increasing flux through the hexosamine biosynthesis pathway (HBP) [3, 16]. The first committed step in the HBP is the conversion of fructose-6-phosphate to glucosamine-6-phosphate. This reaction is catalyzed by the rate-limiting enzyme: glutamine:fructose-6-phosphate amido-transferase (GFAT). Adenovirus directed over-expression of GFAT promotes a significant increase in ER stress markers, as well as lipid accumulation, inflammatory gene expression and apoptotic signaling [17]. Furthermore, vascular and hepatic cells treated with glucosamine in culture show an increase in ER stress markers and activation of pro-atherogenic pathways [3, 18]. These studies suggest that glucose flux through the HBP plays an important role in initiating ER stress and pro-atherogenic pathways. Further *in vivo* studies are required to fully understand the role of the HBP in hyperglycemia-induced atherosclerosis.

In this study, we hypothesized that glucosamine-supplementation would induce phenotypic changes similar to those observed in STZ-induced hyperglycemia in apoE^{-/-} mice, including elevated levels of ER stress and accelerated atherosclerosis, without significantly altering blood glucose or insulin levels. As such, this model will potentially

provide a means to more specifically examine one consequence of hyperglycemia and its downstream effects on pro-atherogenic pathways and lesion development.

2. Materials and methods

2.1 Mouse model

Five-week-old female apoE^{-/-} mice (B6.129P2-apoE^{tm1Unc}) were randomly divided into treatment groups (n = 12 per group) and fed a standard chow diet (TD92078; Harlan Teklad, Madison, WI). To induce hyperglycemia, one group received multiple (10) low-dose intraperitoneal injections (40 mg/kg body weight) with streptozotocin (STZ; Sigma Aldrich, Oakville, ON) as previously described [2, 3]. Other treatment groups were supplemented, via drinking water, with either 5% w/v mannitol (M9647; Sigma), 5% w/v D-(+)-glucosamine hydrochloride (G4875; Sigma) or 5% w/v D-(+)-glucosamine hydrochloride + 625 mg/kg sodium valproate (P4543; Sigma). The drinking water of the control group was not supplemented. All mice were sacrificed at 15 weeks of age and their blood and tissues were collected for analysis. All mice had unrestricted access to both food and water throughout the study. The McMaster University Animal Research Ethics Board approved all procedures.

2.2 Neutral lipid staining

The accumulation of neutral lipids in mouse liver tissue was determined by Oil Red O staining. Fresh frozen liver cross-sections mounted on glass slides were fixed with 37% formaldehyde and then stained with a working solution of 0.5% w/v Oil Red O

(00625; Sigma) in 60% isopropanol/dH₂O. Mayer's hematoxylin solution (MHS32; Sigma) was used to stain and visualize the cell nucleus. Images were captured with a CCD color camera (DP72; Olympus) and analyzed using imageJ software. Staining was digitally quantified and normalized to cross sectional area.

2.3 Immunoblot analysis

Monoclonal antibodies to GADD153/CHOP (sc-7351) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PDI (SPA-891) was purchased from StressGen Biotechnologies (Victoria, BC). RL2 (MA1-072) antibodies against O-GlcNAc were purchased from Affinity Bioreagents (Golden, CO). Anti- β -actin (AC-74) antibodies were purchased from Sigma Aldrich. Total protein lysates from liver tissues were quantified by Bradford assay, solubilized in 4x SDS-PAGE sample buffer and separated on SDS-polyacrylamide gels under reducing conditions. After 1 hour room temperature incubation with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies (Life technologies, Carlsbad, CA), the nitrocellulose membranes were developed using the Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA). Band densities were quantified using imageJ.

2.4 Histochemistry

Mice were euthanized, hearts were flushed with 1xPBS, and perfusion-fixed with 10% neutral buffered formalin. After harvesting the organs, the hearts (including the

aortic root) were cut transversely and embedded in paraffin. Serial sections (4 μm) of aortic root were collected on pre-coated glass slides. Sections were stained with hematoxylin and eosin for measurement of lesion size and necrotic area [18, 19]. The necrotic area was identified as acellular regions (hematoxylin negative) within the atherosclerotic plaque with a minimum size exclusion of 500 μm^2 ; this value ensured single lipid engorged macrophage/foam cells were excluded.

The Vectastain ABC System (Vector Laboratories, Burlingame, CA) was used for immunohistochemical staining. Sections were stained with the primary antibody CTD110.6 (MMS-248R) for O-GlcNAc (Convance Inc., Emery, CA) or KDEL (SPA-827) for Grp78/94 (Stressgen, Victoria, BC) at 4°C overnight. The appropriate biotinylated secondary IgM (BA-2020) or IgG (BA-2000) antibody and Nova Red (all from Vector Laboratories, Burlingame, CA) were used to visualize the stained sections. Non-specific staining was controlled for using pre-immune IgM (M5905, Sigma-Aldrich) or IgG (sc-2025, Santa Cruz, CA) instead of the primary antibody (Suppl. Figure 1). Images were captured with a CCD color camera (DP72; Olympus) for analysis and positively stained regions were quantified with imageJ software using the color deconvolution plugin.

2.5 Statistical analysis

The results are presented as the mean \pm standard error. A one-way analysis of variance and Tukey HSD test was used to determine significant differences between treatment groups. Probability (P) values of <0.05 were considered significant.

2.6 Supplemental materials and methods

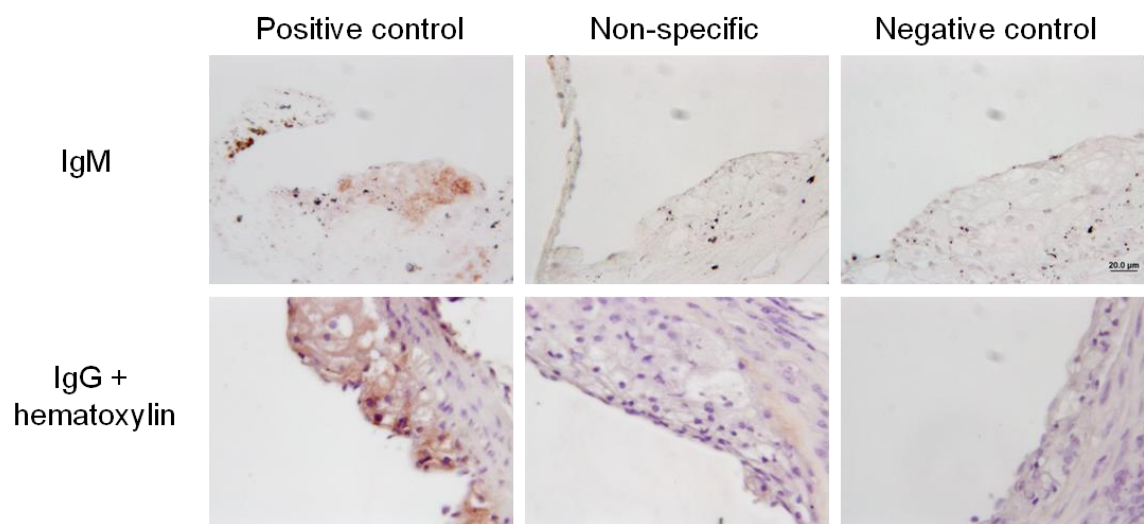
2.6.1 Plasma analysis

Whole-blood glucose levels were measured using a DEX glucometer (Bayer, Toronto, ON). Plasma glucose and lipid levels were determined in non-fasted mice using the colorimetric diagnostic kits for glucose, total cholesterol and triglycerides purchased from Thermo Scientific (Rockford, IL). Insulin levels were determined in non-fasted mice using the insulin ELISA kit from Crystal Chem Inc. (90080; Downers Grove, IL). Plasma lipoproteins were separated by fast protein liquid chromatography (FPLC), and total cholesterol was measured in each fraction using an enzymatic kit (TR13421; Thermo Scientific, Rockford, IL).

2.6.2 Oral glucose/glucosamine tolerance test

An oral glucose tolerance test and an oral glucosamine tolerance test were performed on fasted mice. Whole-blood glucose measurements were taken from 12 hour fasted mice before glucose (2 g/kg body weight) or glucosamine (2 g/kg body weight) was administered orally. Blood glucose levels were then measured periodically over 180 minutes.

Supplemental Figure 1. **Control stains for the immunohistochemical procedure.** Aortic sections positively stained with CTD110.6 (IgM) or KDEL (IgG) antibodies are shown alongside a non-specific control (pre-immune IgM or IgG) and a negative control (primary antibody absent).



Beriault *et al.* Atherosclerosis 2011

3. Experimental results

3.1 Effects of glucosamine-supplementation and STZ-induced hyperglycemia in apoE^{-/-} mice

Multiple low dose injections of STZ induce a significant, and chronic, hyperglycemic state in apoE^{-/-} mice. At 15 weeks of age blood glucose levels in STZ-injected apoE^{-/-} mice were 26.9 ± 2.1 mM compared to 7.4 ± 0.3 mM in apoE^{-/-} mice injected with citrate buffer ($P < 0.01$, Suppl. Table 1). Consistent with previous studies, hyperglycemic mice showed no significant change in total plasma cholesterol (Suppl. Table 1) or lipid profile relative to controls (Suppl. Figure 2) at 15 weeks of age [3, 19].

Supplementation of drinking water with 5% glucosamine (w/v) equated to approximately 0.5 g/kg body weight per day or 0.3% of the total calorie intake per day. To control for possible non-specific effects of supplementing drinking water, 5% mannitol (w/v) was given to a separate group of apoE^{-/-} mice. At the concentrations tested, neither glucosamine nor mannitol had significant effects on fed or fasting blood glucose levels relative to age-matched mice drinking non-supplemented water (Suppl. Table 1; Suppl. Figure 3). Furthermore, glucosamine or mannitol supplementation did not significantly affect body weight, liver weight, plasma triglycerides, insulin, total cholesterol (Suppl. Table 1), or the cholesterol lipid profile (Suppl. Figure 2) when compared to the control. Preliminary experiments using higher concentrations of glucosamine appeared to induce a slight, non-significant reduction in blood glucose concentrations (data not shown). All subsequent experiments were carried out with 5% (w/v) supplementation.

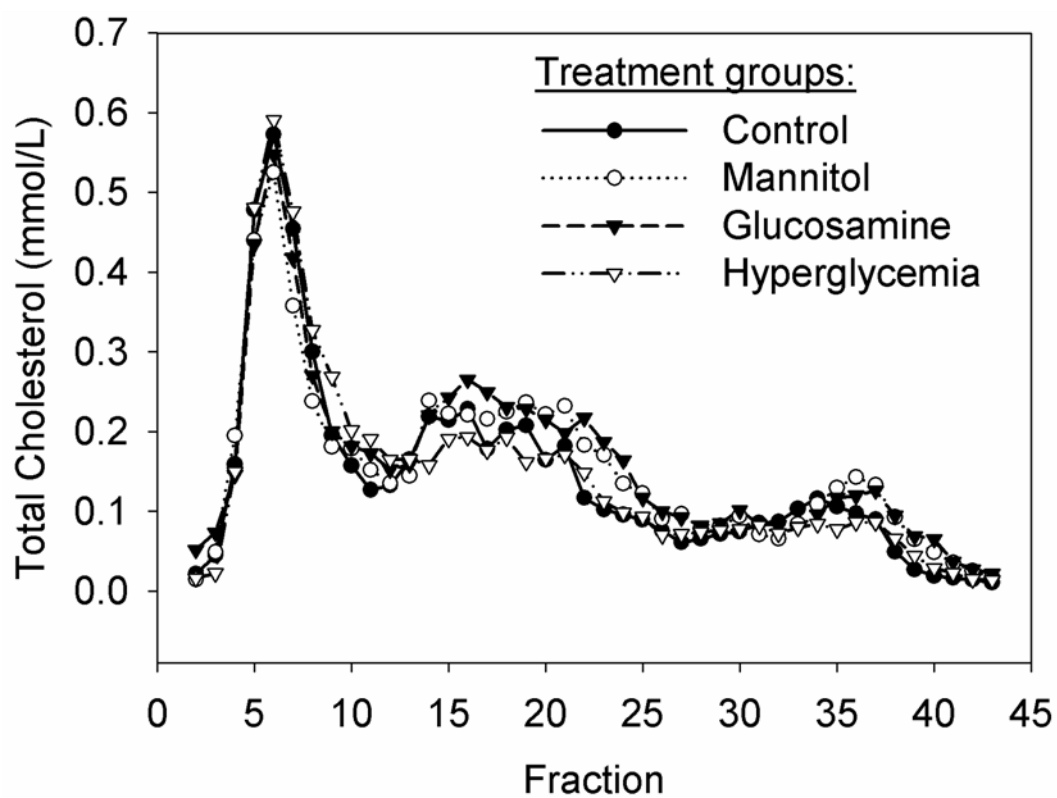
An oral glucose tolerance test (OGTT) was performed to examine possible effects of glucosamine supplementation on glucose tolerance. Fourteen week old glucosamine- and mannitol-supplemented mice were fasted overnight and then gavaged with 2 g/kg body weight glucose. Blood glucose levels were monitored at 30 minute intervals for three hours. No significant differences in glucose response were observed between groups (Suppl. Figure 3A). To examine the acute effects of glucosamine and mannitol on blood glucose levels we gavaged fasted apoE^{-/-} mice with 2 g/kg body weight glucosamine or mannitol. Blood glucose levels were monitored at 30 minute intervals. There were no significant differences in glucose response relative to the control (Suppl. Figure 3B).

Supplemental Table 1. **The general effects of glucosamine- and mannitol-supplementation, and STZ-induced hyperglycemia in 15 week old female apoE^{-/-} mice.** *P<0.05, **P<0.01 relative to the control group; #P<0.05, ##P<0.01 relative to the glucosamine-supplemented group. na, data not available.

apoE ^{-/-} mice	Treatment Group			
	Control	Mannitol	Glucosamine	Hyperglycemia
<i>n</i>	12	12	12	6
Body mass (g)	20.3 ± 0.3	19.9 ± 0.5	20.2 ± 0.5	18.1 ± 0.4 ***
Liver mass (g)	0.87 ± 0.03	0.75 ± 0.03	0.86 ± 0.04	0.96 ± 0.05 *#
Blood glucose (mmol/L)	7.4 ± 0.3	7.1 ± 0.3	7.1 ± 0.3	26.9 ± 2.1 ***
Plasma				
<i>n</i>	8	8	8	6
Triglycerides (mmol/L)	0.75 ± 0.1	0.46 ± 0.06 *	0.62 ± 0.1	1.14 ± 0.07 ***
Cholesterol (mmol/L)	9.2 ± 0.7	7.5 ± 1.2	7.5 ± 0.7	9.3 ± 1.0
Insulin (ng/ml)	0.38 ± 0.05	0.33 ± 0.02	0.43 ± 0.05	na

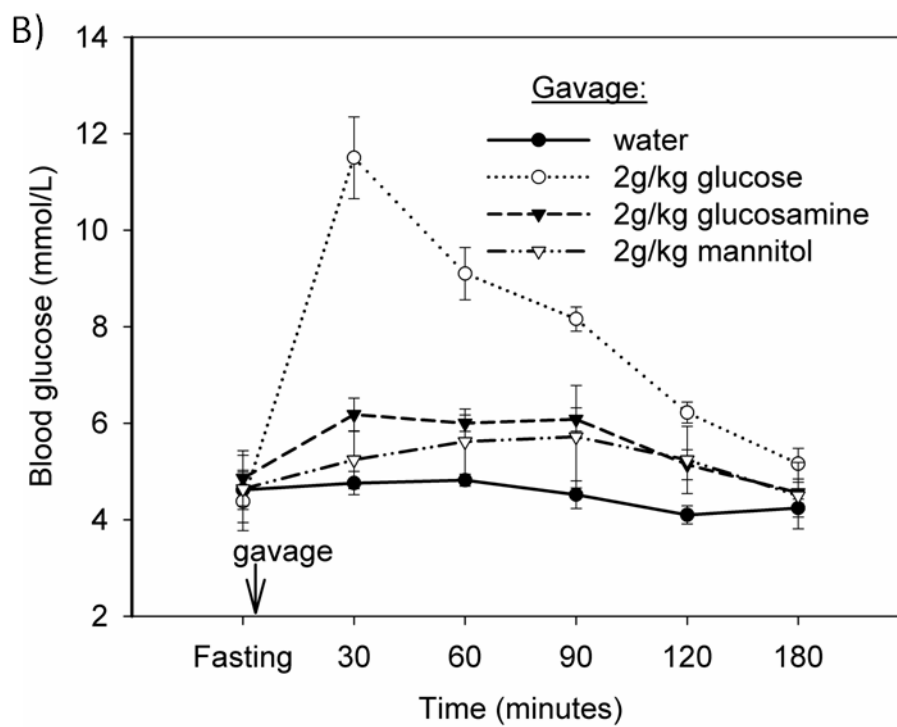
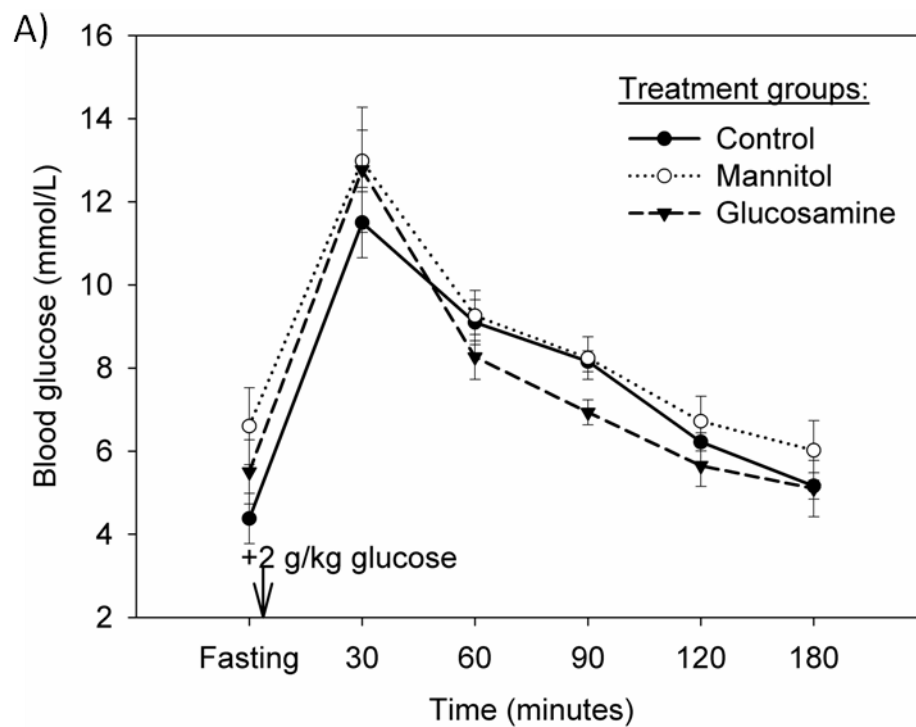
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Supplemental Figure 2. **The effect of glucosamine supplement on the plasma lipid profile.** Plasma from 15 week old female apoE^{-/-} mice, from each of the treatment groups (n=4), was resolved by FPLC. Total cholesterol in each fraction was determined and plotted.



Beriault *et al.* Atherosclerosis 2011

Supplemental Figure 3. **The effect of an oral glucose or glucosamine challenge on blood glucose levels.** A) Fifteen week old apoE^{-/-} mice, supplemented with either 5% glucosamine or 5% mannitol for 9 weeks, were fasted overnight, and then given an oral glucose (2 g/kg body weight) challenge. Blood glucose levels were measured at 30 minute intervals over the next 180 minutes (n=6). B) Normoglycemic apoE^{-/-} mice were fasted overnight, and then gavaged with either 2 g/kg glucose, 2 g/kg glucosamine, or 2 g/kg mannitol as indicated. Blood glucose levels were measured over 180 minutes. Average blood glucose concentration at each time point is indicated (n=5/treatment group).

Beriault *et al.* Atherosclerosis 2011

3.2 STZ-induced hyperglycemia and glucosamine-supplementation increase intracellular glucosamine levels

To identify changes in intracellular glucosamine concentration, we monitored the extent of *O*-N-acetylglucosamine (O-GlcNAc) modification of cellular proteins by immunohistochemical and immunoblot analysis, as previously described [20]. STZ-induced hyperglycemic mice and glucosamine supplemented apoE^{-/-} mice had similar levels of O-GlcNAc modification, that were significantly elevated (5-7 fold, $P < 0.05$) over controls. O-GlcNAc accumulation was evident in both hepatic tissue and in macrophage foam cells of the atherosclerotic lesion (Figure 1).

3.3 Glucosamine-supplementation is associated with activation of the UPR in apoE^{-/-} mice

Previous studies in our lab have shown that STZ-induced hyperglycemic mice show indications of increased ER stress and UPR activation in the artery wall and hepatic tissue [19]. To determine if glucosamine-supplementation can promote ER stress, we stained aortic root and liver tissues with an antibody specific for the ER resident chaperones, Grp78 and Grp94. The results show a significant 4 fold increase in Grp78/94 levels in both aortic root lesions and liver tissue from hyperglycemic and glucosamine-supplemented mice relative to controls (Figure 2A,B). Immunoblot analysis of total liver protein lysates indicates a significant increase in the levels of two additional UPR proteins, PDI and GADD153/CHOP, in both the glucosamine-supplemented and STZ-hyperglycemic mice (Figure 2C,D).

Figure 1. Identifying changes in intracellular glucosamine levels.

A) Immunohistochemical staining of sections of aortic root and liver with an antibody specific for protein-O-GlcNAc (scale bars = 50 μ m). B) The immunohistochemical stains of aortic and liver sections were quantified, normalized by lesion area, and presented as fold change (* $P < 0.05$, ** $P < 0.01$, $n = 5$). C) Immunoblot analysis of O-GlcNAc modified proteins in lysates prepared from livers from each of the treatment groups. The nuclear pore protein, p62, is indicated. Blots were stained for β -actin as a loading control. D) The levels of p62-O-GlcNAc were quantified, normalized to β -actin and presented as fold change (* $P < 0.05$, $n = 7$).

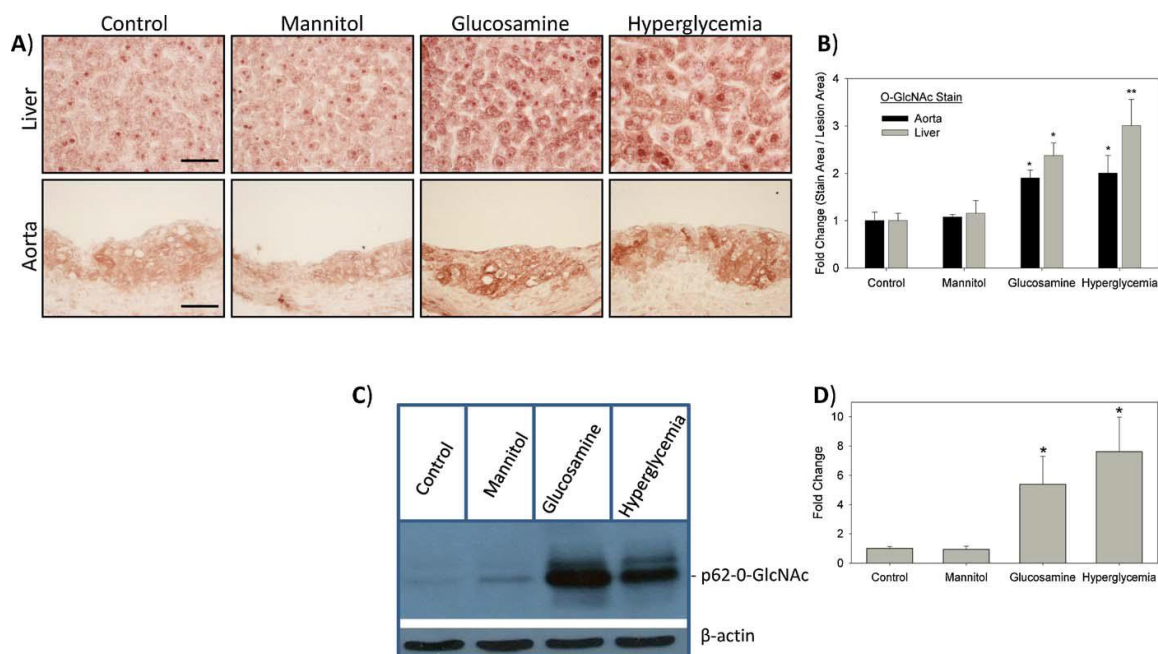
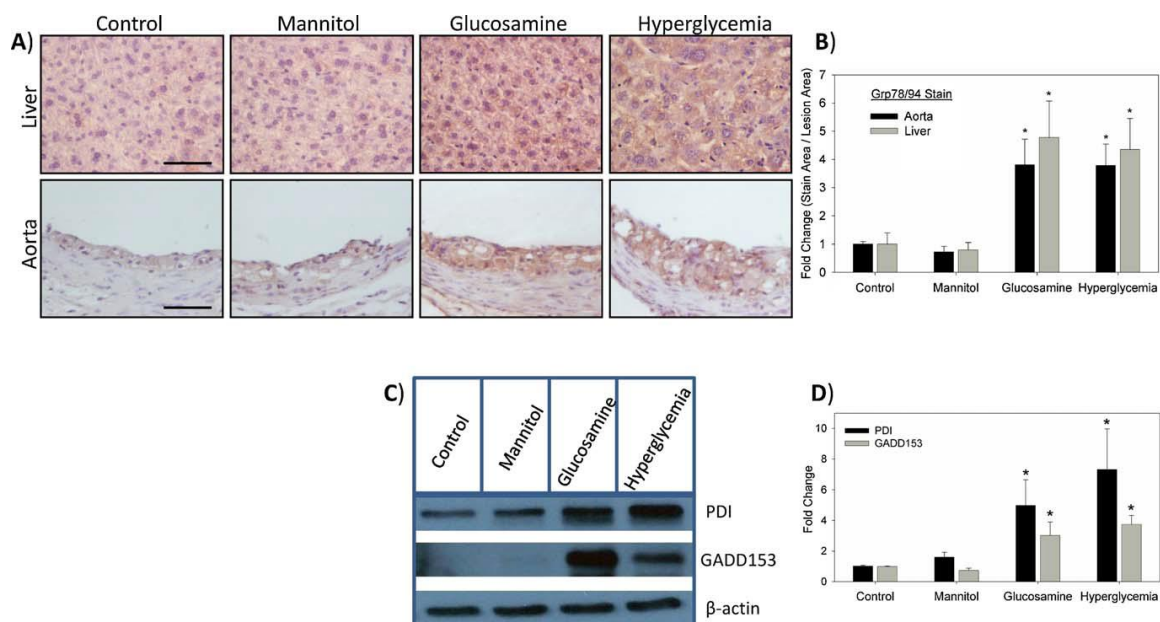
Beriault *et al.* Atherosclerosis 2011

Figure 2. Activation of the ER stress response. A) Sections of aortic root and liver were immunohistochemically stained with an antibody against Grp78/94 (red) and counterstained with hematoxylin nuclear stain (blue) (scale bars = 50 μ m). B) Immunohistochemical stains were quantified, normalized by lesion area, and represented as fold change (* $P < 0.05$, $n = 5$). C) Immunoblot analysis of total protein lysates from liver stained with antibodies specific for PDI or GADD153/CHOP. Blots were probed with an antibody against β -actin as a loading control. D) PDI and GADD153/CHOP protein levels were determined, normalized to β -actin, and presented as fold change (* $P < 0.05$, $n = 6$).

Beriault *et al.* Atherosclerosis 2011

3.4 Hepatic lipid accumulation in glucosamine-supplemented mice

Conditions of chronic ER stress have previously been shown to dysregulate lipid metabolism and promote intracellular cholesterol and fatty acid accumulation [13, 14]. To determine the effects of glucosamine-supplementation on hepatic lipid levels, we stained cross-sections of frozen liver tissue for neutral lipids using Oil Red O. The results show that both glucosamine-supplementation and STZ-induced hyperglycemia are associated with an increase in the neutral lipid content within the liver of apoE^{-/-} mice (2.6 ± 0.5 and 3.0 ± 0.9 fold respectively, $P < 0.05$), relative to controls (Figure 3).

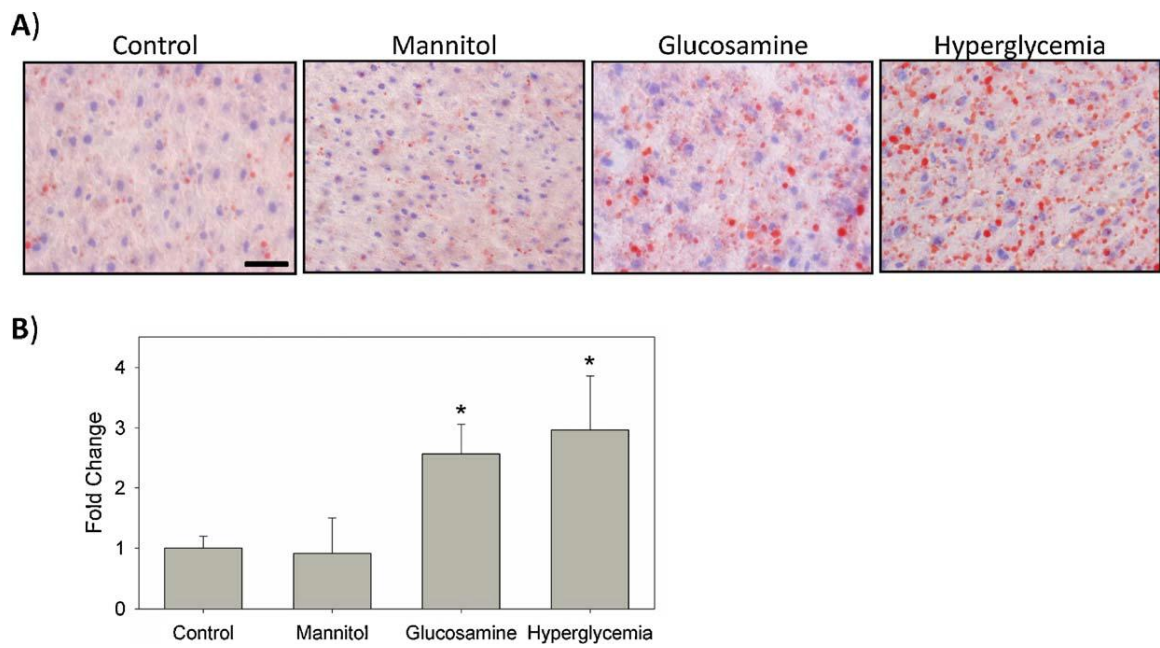
3.5 Glucosamine-supplementation accelerates atherogenesis in an apoE^{-/-} mouse model

At 15 weeks of age, the glucosamine-supplemented mice had significantly larger and more advanced atherosclerotic lesions at the aortic root compared to control or mannitol-supplemented mice (Figure 4A). Lesion area and necrotic area in the lesions from the glucosamine-supplemented group were similar to those observed in STZ-hyperglycemic mice (Figure 4B,C). No significant atherosclerotic lesions were observed in the descending aortas of any of the mouse groups examined (data not shown).

We have previously shown that feeding hyperglycemic mice a diet containing 625 mg/kg sodium valproate can attenuate accelerated atherosclerosis in STZ-induced hyperglycemic mice [19]. We next wanted to determine if sodium valproate would have a similar mitigating effect on atherosclerosis in glucosamine-supplemented mice. At 15 weeks of age, mice supplemented with both 5% glucosamine and 625 mg/kg sodium

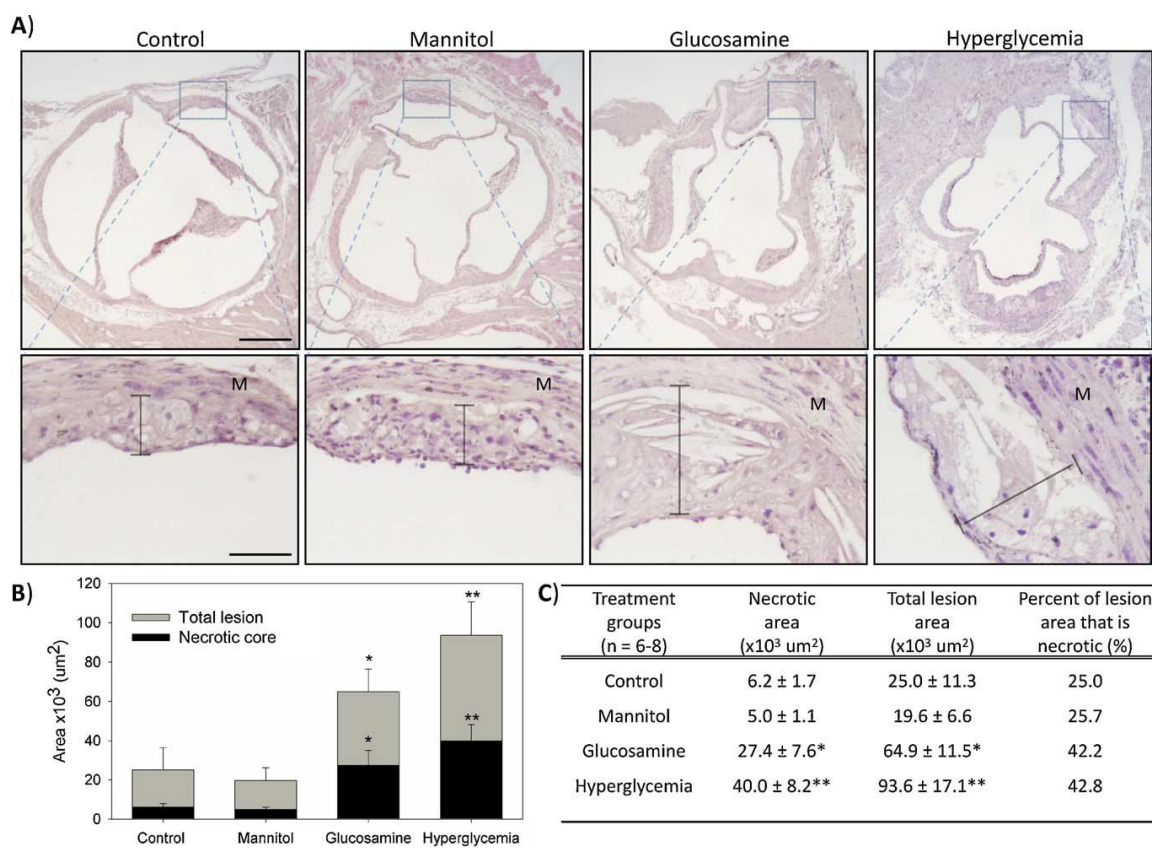
valproate had significantly less necrotic area and lesion area at the aortic sinus relative to mice supplemented with only 5% glucosamine (Figure 5). Furthermore, the glucosamine- and valproate-supplemented mice had significantly less total lesion volume along the ascending aorta relative to the 5% glucosamine group (Figure 5B,C). There was no significant difference in necrotic area, lesion area, and total lesion volume between the glucosamine/valproate-supplemented group and the control (Figure 5B,C).

Figure 3. Measuring hepatic lipid concentration A) Sections of liver from each of the treatment groups were stained for neutral lipids using Oil Red O (red) and counterstained with hematoxylin nuclear stain (blue). B) Lipid area was quantified and depicted as fold change relative to the control (* $P < 0.05$, $n = 3$). Scale bar = 200 μm .



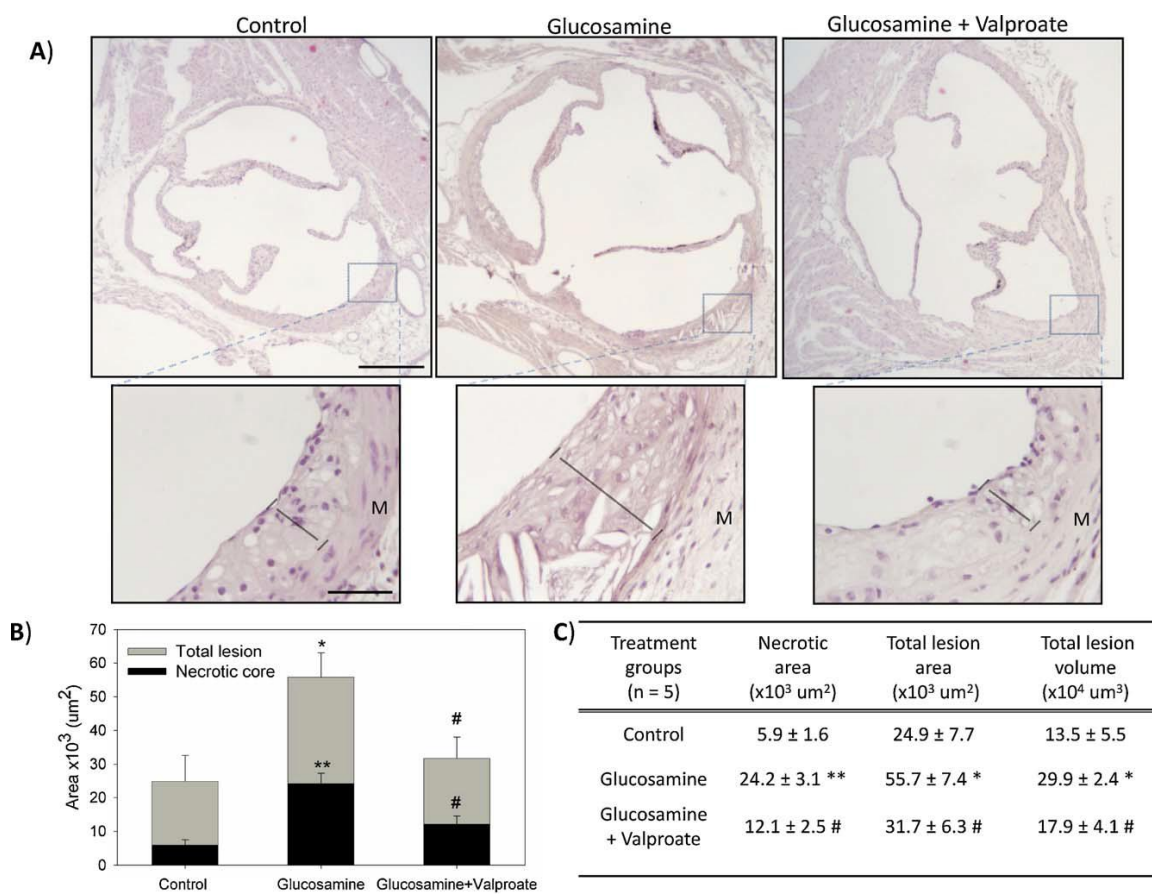
Beriault *et al.* Atherosclerosis 2011

Figure 4. **Analysis of atherosclerotic lesion development.** A) Sections of aortic root from each treatment group stained with hematoxylin and eosin showing the entire vessel cross section (scale bar = 500 μm), as well as a close up of the lesion (scale bar = 50 μm), as indicated. The line depicts the width of the lesion, and “M” indicates the medial layer of the vessel wall. B+C) Quantification of the total lesion area, and extent of necrosis, at the aortic sinus (* $P < 0.05$, ** $P < 0.01$ relative to the control group, $n = 6-8$).



Beriault *et al.* Atherosclerosis 2011

Figure 5. Effect of valproate on atherosclerotic lesion size. Atherosclerosis lesion development was examined in 15 week old apoE^{-/-} mice that received the glucosamine supplement in the absence or presence of 625 mg/kg valproate. A) Sections of aortic root from each treatment group stained with hematoxylin and eosin (top scale bar = 500 μ m, bottom scale bar = 50 μ m). B+C) Quantification of the total lesion area and the necrotic area at the aortic sinus as well as the total lesion volume along the ascending aorta. *P<0.05, **P<0.01 relative to the control group; #P<0.05 relative to the glucosamine group, n=5.

Beriault *et al.* Atherosclerosis 2011

4. Discussion

In this study we show that glucosamine supplementation promotes hepatic lipid accumulation and accelerated atherosclerosis in apoE^{-/-} mice. In this model, glucosamine-supplementation does not cause detectable alterations in the lipid profile, blood glucose concentrations, glucose tolerance or plasma insulin levels relative to controls. Our data show that glucosamine supplementation is associated with a significant increase in hepatic and vascular ER stress level. Treatment with valproate, a small molecule that we and others have shown to block ER stress signaling pathways [19, 21], attenuates glucosamine-induced accelerated atherogenesis. The effects of glucosamine-supplementation closely mimic the pro-atherogenic effects of STZ-induced hyperglycemia in apoE^{-/-} mice. We propose that glucosamine, a metabolite of glucose, is a causative agent in hyperglycemia associated accelerated atherosclerosis and that the mechanism by which glucosamine activates pro-atherogenic processes involves the induction of ER stress.

Glucosamine is a product of the hexosamine biosynthesis pathway. In a typical cell, 1-3% of intracellular glucose is converted to UDP-*N*-acetylglucosamine which is an essential substrate for O- and N-linked protein glycosylation and a component of glycosaminoglycans including heparin. Oral glucosamine is widely used as a supplement to alleviate osteoarthritis or general soreness in the joints; however, the efficacy of glucosamine in this regard remains controversial [22]. Conflicting data from *in vitro* experiments has shown glucosamine to have both pro- and anti-inflammatory effects [23, 24].

In a previous study, Tannock *et al.* directly examined the effect of a human equivalent dose (15 mg/kg/day) of glucosamine on the development of atherosclerosis in a LDLR^{-/-} mouse model [25]. They found that glucosamine accelerated early stage, but not late, atherosclerosis in this model. Other tissues, such as liver, were not examined in this study, and no mechanism of action was determined to explain the effect. Despite the observed early effect on atherosclerosis, the general conclusion of this study was that glucosamine supplementation had no long term detrimental effects and appeared to be safe.

Conditions of hyperglycemia can increase the flux through the HBP, and subsequently increase protein-O-GlcNAc [18, 26]; which is indicative of the increased intracellular glucosamine concentration [20]. This pathway has been implicated in the development of insulin resistance in cell culture and animal models [16, 27]. In our study, we supplemented apoE^{-/-} mice with 5% (approximately 500 mg/kg/day) glucosamine for 10 weeks. This level is approximately 30 times the concentration used by Tannock *et al.* and substantially higher than the equivalent dose in a human glucosamine supplement. We did not detect any indications of reduced glucose tolerance in the supplemented mice however, the intracellular levels of O-GlcNAc was significantly elevated to a level that was indistinguishable from the increase in O-GlcNAc observed in STZ-induced hyperglycemic mice. Therefore, we conclude that this supplement matches the increase in intracellular glucosamine concentration that corresponds to severe hyperglycemia.

Glucosamine has long been known to be a potent inducer of ER stress [28, 29]. Our lab has previously shown that elevated concentrations of glucose and glucosamine

can induce ER stress in cultured HepG2 cells [30], and in vascular cell types including monocytes [18], human aortic smooth muscle cells [18] and endothelial cells [3]. It is becoming increasingly evident that ER stress plays an active role in the development of atherosclerosis. Previous work has shown that glucosamine-induced ER stress can promote lipid accumulation (SREBP) and the activation of inflammatory pathways (NF- κ B, IL-1 α , IL-8) in these cell types [17]. Data from studies in animal models suggest that ER stress plays a direct role in accelerating atherosclerosis in hyperhomocysteinemic, obese, and hyperglycemic mice [3, 6, 9, 31]. In this study hepatic and vascular ER stress levels were similar in STZ-induced hyperglycemic and glucosamine-supplemented mice.

We have previously shown that valproate, a small branch chain fatty acid, can disrupt ER stress signaling pathways and attenuate the downstream complications of ER stress without affecting the UPR. In cultured cells, valproate can protect against ER stress induced lipid accumulation [30]. In hyperglycemic apoE^{-/-} mice, valproate treatment significantly slows atherogenesis and the development of hepatic steatosis [19]. Glucosamine-supplemented apoE^{-/-} mice treated with valproate showed a significant decrease in atherosclerotic lesion development relative to the glucosamine group. These findings suggest that glucosamine-induced ER stress promotes atherogenesis.

Together the results of this study highlight the potential of glucosamine-supplementation as a model of ER stress-associated accelerated atherosclerosis. Furthermore, this model may illuminate the mechanisms by which diabetes and hyperglycemic promote atherogenesis. The major advantage of this model is that, unlike STZ-induced hyperglycemia, glucosamine supplementation does not significantly affect

blood glucose levels, glucose tolerance or insulin levels, but it does promote the downstream accelerated development of atherosclerosis. Furthermore, we did not observe any obvious indications of insulin resistance. These findings suggest that, glucose can act indirectly, through a glucosamine intermediate, to promote atherosclerosis, and that hypoinsulinemia is not essential to accelerate plaque development. Furthermore, by supplementing with glucosamine, we are able to by-pass the glycolytic pathway and potentially avoid the mitochondrial oxidative stress response that may occur in a hyperglycemic state.

Additional studies are required to better understand the mechanisms and pathways involved in accelerated atherogenesis. For example, it is not known how elevated concentrations of glucosamine cause ER stress and activate the UPR. Data from Qiu *et al.* show that in cultured HepG2 cells, glucosamine can disrupt the N-linked glycosylation of apoB-100 [29]. Glycation of proteins is an important and essential step in proper protein folding. Therefore, it is possible that glucosamine induces ER stress by generally disrupting the N-linked glycation of proteins in the ER.

Furthermore, if hyperglycemia and glucosamine-associated ER stress promote atherogenesis, does this occur directly and locally, in the vascular wall or indirectly through effects on other tissues? This report and others have clearly shown that the liver is highly susceptible to ER stress and ER stress-associated lipid metabolism [32]. In this study, we see no detectable difference in the lipid profile between control, hyperglycemic and glucosamine-supplemented mice at 15 weeks of age, however we cannot rule out the effect of subtle changes in lipoprotein metabolism and lipid transport. At later time

points, STZ-hyperglycemic mice do develop relative dyslipidemia that likely contributes to the progression of atherogenesis [18]. Future studies addressing these and other questions will help to delineate the mechanisms and relevance of hyperglycemia-induced ER stress in the accelerated development of atherosclerosis.

5. Conflict of interest statement

The authors have no conflicts of interest to declare.

6. Acknowledgements

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4 INVESTIGATION OF THE EFFECTS OF GLUCOSAMINE ON LIPID-LINKED OLIGOSACCHARIDE BIOSYNTHESIS *IN VITRO* AND *IN VIVO*

4.1 Rationale

Currently, it is unknown how glucosamine activates the UPR. Previous research has shown that UDP-N-acetyl-glucosamine is the initial substrate for lipid-linked oligosaccharide (LLO) synthesis, and LLOs are substrates for N-linked glycosylation [107]. Increasing the intracellular concentrations of glucosamine may induce ER dysfunction by interfering with the glycosylation of nascent proteins, and thus increasing the propensity of proteins to fold incorrectly. Although glucosamine is also a substrate for O-linked glycosylation, our lab has previously shown that an increase in O-linked glycosylation does not promote ER stress[43]. The goal of this aim is to determine the mechanistic link between glucosamine and ER stress, and ultimately, gain a better understanding of the molecular and cellular mechanisms linking diabetes to the development and progression of atherosclerosis.

4.2 Hypothesis

Elevated concentrations of glucosamine promote ER stress by interfering with lipid-linked oligosaccharide (LLO) biosynthesis, and thereby disrupting N-linked glycosylation and protein folding.

4.3 Objectives

- 1 Examine the effects of glucosamine on LLO biosynthesis *in vitro* and *in vivo* using fluorescence-assisted carbohydrate electrophoresis (FACE).
- 2 Examine the relationship between disruptions in LLO biosynthesis and the up-regulation of ER stress markers.
- 3 Conduct western blots to determine the effects of glucosamine on protein levels/N-glycosylation.

Glucosamine induces ER stress by disrupting lipid-linked oligosaccharide biosynthesis and N-linked protein glycosylation

Abstract

Objectives: To determine the effects of glucosamine on lipid-linked oligosaccharide (LLO) biosynthesis, and to investigate the potential role of LLO disruptions in ER stress activation and atherogenesis.

Results: The LLO biosynthesis pathway was significantly disrupted by the addition of 1-5 mM glucosamine to MEF cells. These disruptions were time and dose dependent and occurred well before changes in mRNA expression of ER stress markers. 4-phenylbutyric acid, a chemical chaperone, was able to alleviate ER stress, as shown by a decrease in ER stress markers, but was unable to restore LLO biosynthesis. Two other ER stress inducing agents, DTT and Thapsigargin, had no significant effect on LLO levels at 4 hours treatment. Glucosamine supplemented apoE^{-/-} mice had elevated levels of LLO intermediates, ER stress and significantly larger and more advanced atherosclerotic lesions compared to the control mice. Our data suggest that elevated concentrations of glucosamine induce ER stress by interfering with lipid-linked oligosaccharide biosynthesis and N-linked glycosylation.

Conclusion: Glucosamine-supplementation causes disruptions in LLO biosynthesis and promotes ER stress and accelerated atherosclerosis in apoE^{-/-} mice. These findings support a model by which conditions of hyperglycemia promote vascular complications via a glucosamine-intermediate.

*Currently unpublished manuscript

1. Introduction

Emerging research has implicated the endoplasmic reticulum (ER) stress response as a plausible mechanism for the accelerated development of atherosclerosis.

Cardiovascular risk factors including dyslipidemia [1, 2], obesity [3], cigarette smoke [4], hypertension [5] and hyperhomocysteinemia [6, 7] have each been associated with elevated levels of ER stress and/or UPR activation. In mouse models, we and others have shown that hyperglycemia is associated with ER stress and the activation of pro-atherogenic pathways. Evidence suggests that the hexosamine biosynthetic pathway plays a role in this process [8-11].

1.1 Hexosamine biosynthesis pathway and endoplasmic reticulum stress

In cultured cells, elevated concentrations of glucose or glucosamine have been shown to induce ER stress by increasing flux through the hexosamine biosynthesis pathway (HBP) [8, 12]. The HBP converts the glucose metabolite: fructose-6-phosphate, to glucosamine-6-phosphate as the first committed step. This conversion is catalyzed by the rate-limiting enzyme: glutamine:fructose-6-phosphate amidotransferase (GFAT). The end product of the HBP results from a multi-step conversion of glucosamine-6-phosphate to UDP-N-acetyl-glucosamine (UDP-GlcNAc); which is a substrate for both O-linked and N-linked glycosylation. While elevated concentrations of glucosamine can increase O-linked glycosylation, our lab has shown that this in itself is not sufficient to promote ER stress [8]. It has been shown that altering glucosamine levels can cause defects in N-glycosylation of ApoB-100 [13] and GRP94 [14]. N-linked glycosylation can play an

essential role in protein folding and the disruption of this process, with tunicamycin for example, promotes ER stress [15, 16].

1.2 Lipid-linked oligosaccharide biosynthesis and N-linked glycosylation

N-linked glycosylation is also important for protein trafficking, stability and function [17]. The N-linked glycosylation of proteins occurs co- or post-translationally in the lumen of the ER. In this process, a large mannose-rich oligosaccharide is transferred onto specific asparagine residues, in the context Asn-X-Ser/Thr, of the polypeptide by oligosaccharyltransferase (OST) [18]. In most eukaryotes, the oligosaccharide is formulated sequentially with the addition of specific monosaccharides (glucose, mannose or N-acetyl-glucosamine) to a pyrophosphate-dolichol (P-P-Dol) on the ER membrane (see Figure 2a). This process is initiated on the cytosolic side of the ER membrane, where UDP-GlcNAc is utilized to transfer GlcNAc-1-P to dolichol phosphate (P-Dol) by GlcNAc-1-P transferase (GPT). GlcNAc-P-P-Dol is further sequentially modified by addition of another GlcNAc (from UDP-GlcNAc) and five mannose residues (from GDP-mannose) to form $M_5\text{-Gn}_2\text{-P-P-Dol}$. A phospholipid transporter flips $M_5\text{-Gn}_2\text{-P-P-Dol}$ to the luminal side of the ER membrane where oligosaccharide biosynthesis continues with the addition on four more mannose residues (from mannose-P-Dol) and three glucose residues (from glucose-P-Dol). The full length form of these lipid-linked oligosaccharides (LLOs) is a branched chain that consists of 3 glucose, 9 mannose, 2 N-acetyl-glucosamine residues, and a diphosphate dolichol (known as $G_3M_9\text{Gn}_2\text{-P-P-Dol}$). Disruptions in LLO biosynthesis can have a direct effect on N-linked glycosylation, and may impede protein folding. For example, the antibiotic tunicamycin (a UDP-GlcNAc

analog) inhibits the enzyme involved in the first step of LLO biosynthesis, GPT, thereby blocking LLO biosynthesis and N-linked glycosylation, resulting in protein misfolding and ER stress [17, 19, 20]. Currently, it is not well understood how glucosamine promotes ER stress and activates the unfolded protein response (UPR). As UDP-GlcNAc is a downstream product of glucosamine, and is an important substrate for LLO biosynthesis, this mechanism may play an important role in UPR activation.

2. Methods

2.1 Cell culture

Mouse embryonic fibroblasts (MEFs) were cultured in media consisting of 1x DMEM (Invitrogen, Burlington, ON) +10% fetal bovine serum (Hyclone, Rockford, IL) and incubated in 5% CO₂ at 37°C. Cells were grown to ~90% confluence in either T-75 flasks for LLO analysis or 6 well dishes for western blot and RT-PCR analysis. Cells were treated with either 0-5 mM D-(+)-glucosamine (GlcN; G4875 Sigma), 4 µg/mL tunicamycin (TM; T7765, Sigma), 1 mM dithiothreitol (DTT; D0632, Sigma), or 500 nM thapsigargin (Thaps; T9033, Sigma) for the indicated time intervals and harvesting protocols listed below.

2.2 RT-PCR

Total RNA was isolated from MEF cells using the QIAamp RNeasy Mini Kit (Qiagen, Mississauga, ON). 1 µg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantification of mRNA levels was performed by real-time PCR of cDNA using SYBR

Green ER Master Mix (Invitrogen) and the ABI 7300 system and software (Applied Biosystems). Biological and technical samples were run in triplicate and expression levels were normalized using the β -actin endogenous control. Primers used to amplify ER stress markers included GRP78/BiP: Fwd 5'-ACCTGGGTGGGGAAGACTTT-3' Rev 5'-TCTTCAAATTTGGCCCGAGT-3', C/EBP homologous protein (GADD153/CHOP): Fwd 5'-TATCTCATCCCCAGGAAACG-3' Rev 5'-CTGCTCCTTCTCCTTCATGC-3', and β -actin: Fwd 5'-GGCACCACACCTTCTACAATG-3' Rev 5'-GGGGTGTGTAAGGTCTCAAAC-3'.

2.3 Western blot

Total protein lysates were solubilized in 4x SDS-PAGE gel sample buffer containing DTT and 0.05% bromophenol blue. Protein lysates (50 μ g) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with primary antibodies against GRP78/94 (anti-KDEL; SPA-827, Stressgen Biotechnologies), PDI (SPA-891, Stressgen Biotechnologies) or β -actin (A3854, Sigma). After incubation with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Life Technologies), membranes were developed using the Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA). Blots were imaged and quantified using the Bio-Rad ChemiDoc XRS+ system and ImageLab software.

2.4 Glycoprotein detection

MEF cells were treated with 5 mM GlcN or 4 μ g/mL TM for 18 hours and harvested using 4x SDS sample buffer. The cell lysates were normalized for total protein using a Bradford assay and were separated by gel electrophoresis. The fixated gels were

then stained for glycoproteins using the Pro-Q Emerald 300 Glycoprotein gel stain kit (P21857, Molecular Probes), and for total protein using Coomassie blue R-250 (161-0400, Bio-Rad). Gels were visualized on the XRS+ ChemiDoc imaging system (BioRad) and quantified with ImageLab software.

2.5 Lipid-linked oligosaccharide (LLO) extraction/fluorescence-assisted carbohydrate electrophoresis (FACE)

Cells and tissues were lysed by sonication in 100% methanol. LLOs were extracted from cell and tissue lysates with methanol:chloroform:water (10:10:3) and partially purified with DEAE-cellulose columns as previously described [21-24]. On a selection of our samples, microsomal extractions were done to enrich for the endoplasmic reticulum (ER) before LLO extraction. This involved homogenizing cells/tissue in a 0.25M sucrose solution using a glass piston (3mL per gram of tissue) and spinning the samples at 3000xG for 10 minutes. The supernatant is then spun at 170,000xG for 40 minutes at 4°C yielding a pellet that is resuspended in 100% methanol. Although microsomal extraction was not a required step in the previously published LLO procedure, we added the extra step to ensure our results were indicative of LLOs from the ER.

2.6 Mouse models/tissue processing

ApolipoproteinE-deficient (apoE^{-/-}) mice were randomly divided into two groups (n = 8/group) at five weeks of age and fed a standard chow diet (TD92078; Harlan Teklad, Madison, WI). These mice were supplemented via drinking water with or without 5% w/v D-(+)-glucosamine hydrochloride (G4875, Sigma). All mice were sacrificed at

15 weeks of age and their blood and tissues were collected for analysis. All mice had unrestricted access to both food and water throughout the study. The McMaster University Animal Research Ethics Board approved all procedures.

2.7 Statistical analysis

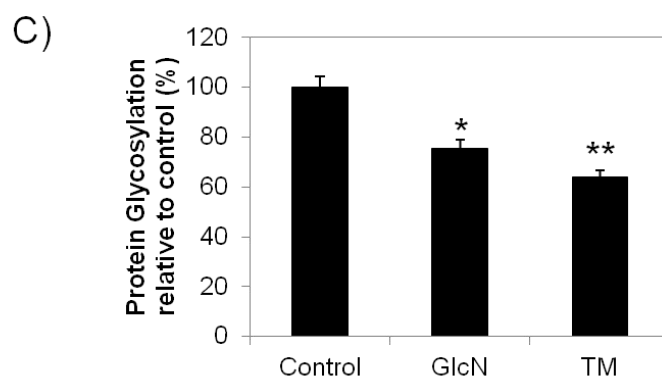
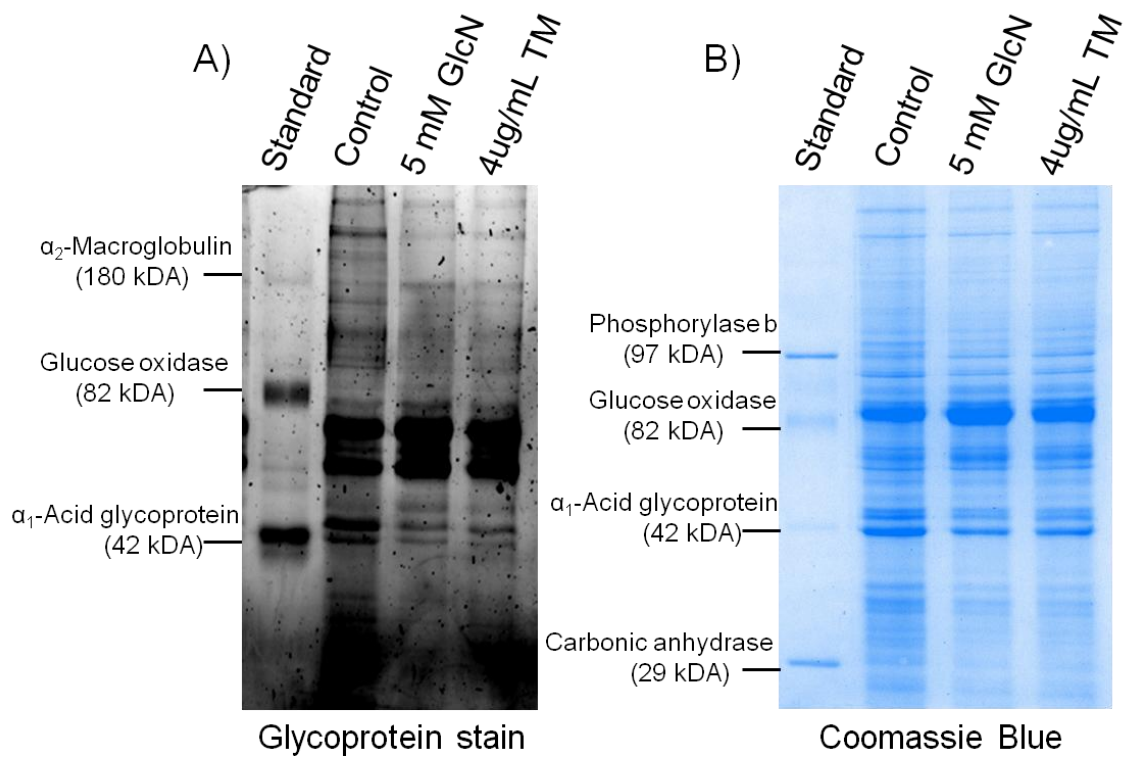
The results are presented as the mean \pm SD. An unpaired Student's t-test was employed to compare data between treated and control samples. A probability value <0.05 was considered statistically significant.

3. Results

3.1 Elevated concentrations of glucosamine disrupt protein glycosylation

It has been previously shown that elevated concentrations of glucosamine are associated with decreased N-linked glycosylation of apoB100 [13]. We hypothesized that excess glucosamine causes global defects in protein N-glycosylation that would be evident throughout the proteome. To test this we treated MEF cells for 18 hours in the presence or absence of 5 mM glucosamine or 4 μ g/mL tunicamycin. Tunicamycin was a positive control in this experiment as it known to block protein N-glycosylation by interfering with LLO biosynthesis [15]. To detect protein glycosylation in our samples, we stained SDS-PAGE gels with the Pro-Q Emerald 300 glycoprotein reagent (Figure 1A). Coomassie blue stain was used to detect total protein levels (Figure 1B). The level of glycosylation for each lane on the gel was quantified by densitometry. Results show that both glucosamine and tunicamycin treated cells had significantly decreased protein glycosylation compared to the untreated control (Figure 1C).

Figure 1. **Elevated concentrations of glucosamine disrupt protein glycosylation.** MEF cells were treated with either 5mM glucosamine (GlcN) or 4ug/mL tunicamycin (TM) for 18 hours. Equal amounts of total protein were resolved by SDS-PAGE. Gels were stained with the Pro-Q Emerald 300 glycoprotein gel staining kit (A), and then re-stained with Coomassie Blue for total protein (B). Entire lanes from the glycoprotein gel were quantified in ImageLab and compared to the control (C). * $p < 0.05$; ** $p < 0.01$; N=3.



3.2 Elevated concentrations of glucosamine disrupt the lipid-linked oligosaccharide biosynthesis pathway

The lipid-linked oligosaccharide (LLO) biosynthesis pathway involves the sequential synthesis of a full length oligosaccharide, G3M9Gn2, which is transferred to specific asparagine residues on nascent proteins in the ER lumen by the enzyme oligosaccharyltransferase (OST) (Figure 2A). To determine the effect of excess glucosamine on the LLO pathway we treated MEF cells with 5 mM glucosamine for 4 hours. Fluorescence-assisted carbohydrate electrophoresis (FACE) was used to visualize LLOs. We found that treating MEF cells with 5 mM glucosamine significantly disrupted LLO biosynthesis. Specifically, in glucosamine treated cells, the full length G3M9Gn2 oligosaccharide was not detectable (Figure 2B) and there was an accumulation of a higher mobility LLO intermediate (indicated by the arrow in Figure 2B). Results from time and dose response experiments indicated that exposure for ≥ 4 hours to concentrations ≥ 1 mM glucosamine were required to increase mRNA expression of specific ER stress markers, GRP78 and GADD153/CHOP (Figure 3A,B). FACE gels showed a similar dose response, as 1 and 5 mM glucosamine significantly disrupted the LLO biosynthetic pathway (Figure 3C,D). Interestingly, the ability of glucosamine to disrupt LLO biosynthesis was observed after only 1 hour treatment (Figure 3C,D); before the induction of GRP78 and GADD153/CHOP expression (Figure 3A,B). These results are consistent with our hypothesis that glucosamine-induced disruption of LLO biosynthesis is a cause of ER stress, rather than a result of ER stress. The 0.2 mM glucosamine treatment did not disrupt the LLO pathway, nor did it elicit an ER stress

response at the 1 or 4 hour time intervals. However 0.2 mM glucosamine appeared to increase the flux through the LLO pathway, as indicated by the larger band corresponding to G3M9Gn2 (Figure 3C,D).

Figure 2. **Glucosamine disrupts lipid-linked oligosaccharide biosynthesis *in vitro*.** A) A schematic of the lipid-linked oligosaccharide (LLO) biosynthesis pathway which produces the glucose(3)-mannose(9)-N-actylglucosamine(2) oligosaccharide that is transferred to the protein by oligosaccharyltransferase (OST). UDP-N-actylglucosamine, a product of the hexosamine biosynthetic pathway (HBP), is the first substrate utilized in LLO biosynthesis. B) MEF cells were treated in the presence or absence of 5mM glucosamine (GlcN) for 4 hours. Results of fluorescent-assisted carbohydrate electrophoresis analysis indicate that the LLO pathway is disrupted in the presence of 5 mM glucosamine. Blue arrows indicate the locations of change relative to the control.

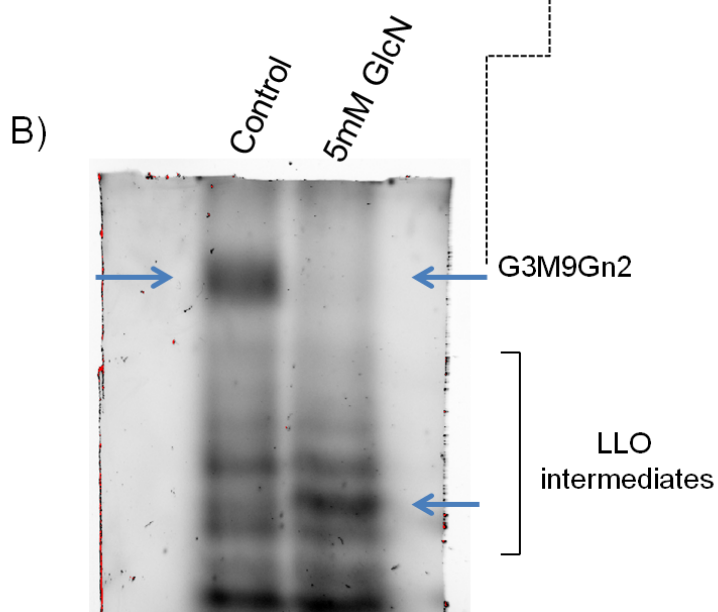
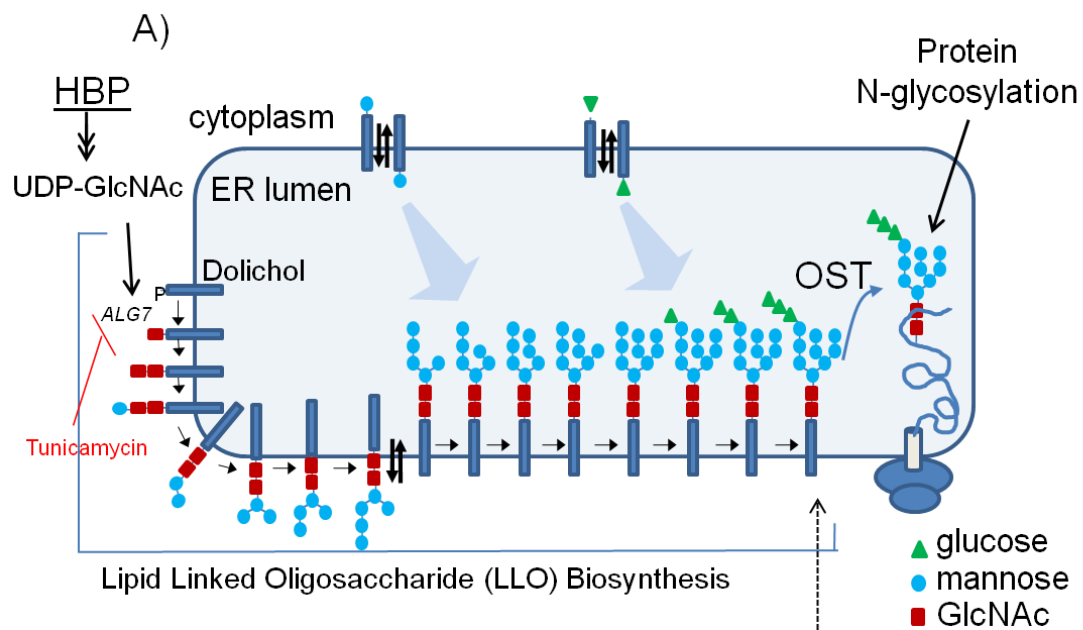
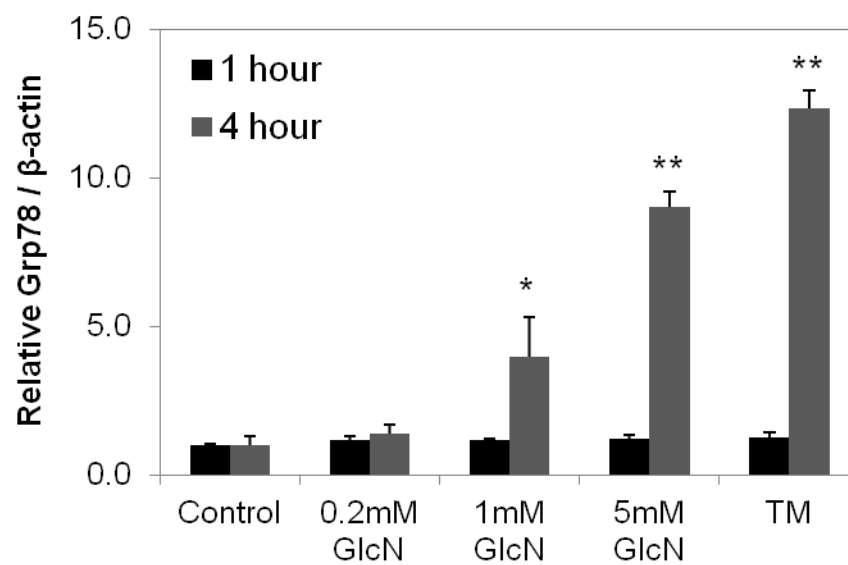


Figure 3. Glucosamine disrupts LLO biosynthesis and induces ER stress in a time and concentration dependent manner in MEF cells. A,B) Realtime-PCR of ER stress markers (GRP78 and GADD153/CHOP) at 1 and 4 hours treatment with increasing doses of glucosamine (0, 0.2, 1, and 5 mM) or 4ug/mL tunicamycin (TM). C,D) FACE gels of the LLO intermediates showing that glucosamine disrupts the pathway in a concentration and time dependent manner. The standard in panel C indicates the location of the G3M9Gn2 band. The standard in panel D is a glucose polymer of 5 to 8 monomers.

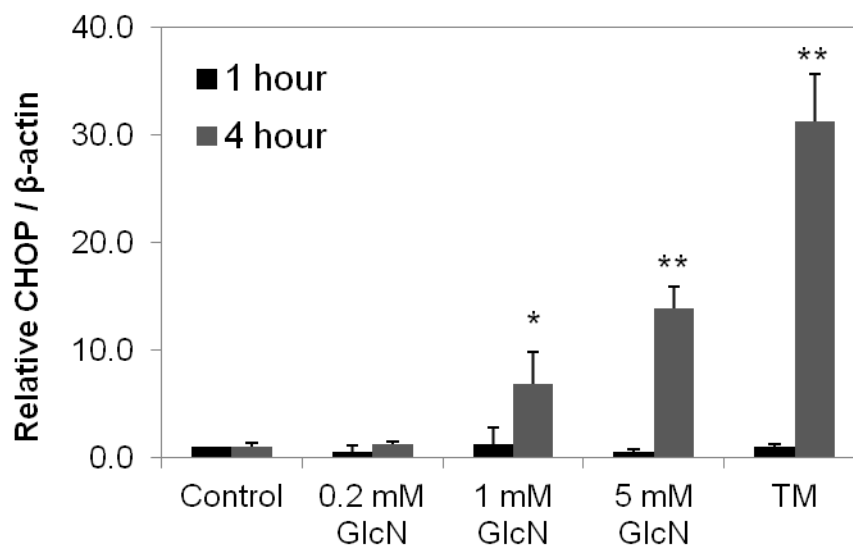
*p<0.05; **p<0.01; N=3.

A) GRP78

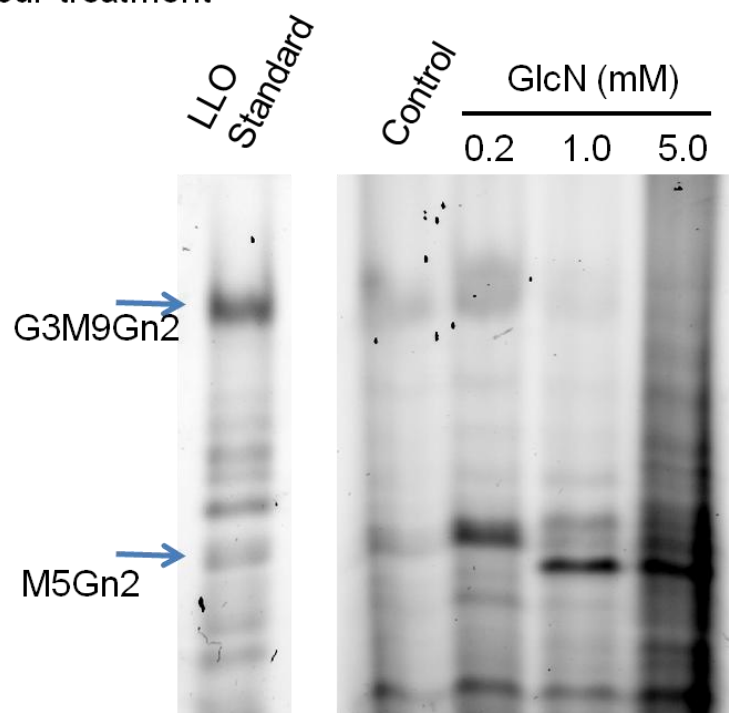
RT-PCR



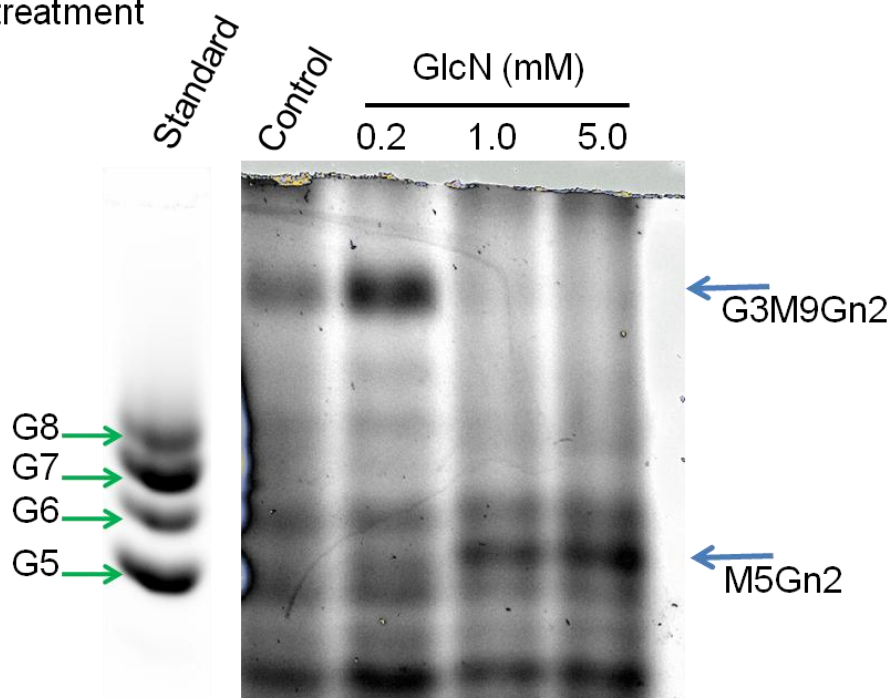
B) CHOP



C) 1 hour treatment



D) 4 hour treatment



*Standards were imaged at a lower exposure setting

3.3 Thapsigargin and DTT induce ER stress but do not disrupt LLO biosynthesis

There are a number of mechanisms by which protein folding can be disrupted and ER stress induced. For example, thapsigargin inhibits SERCA and induces ER stress by disrupting calcium homeostasis [25], whereas DTT disrupts protein disulfide bonds [26]. To determine the effect thapsigargin and DTT have on LLO biosynthesis we treated MEF cells with 1 mM DTT or 0.5 μ M Thapsigargin for 4 hours. RT-PCR analysis showed that GRP78 and GADD153/CHOP mRNA expression was increased and confirmed that the UPR was activated in these cells (Figure 4A). FACE analysis showed there was no change in the amount of LLOs in either treatment group relative to the control (Figure 4B). These results indicated that disrupted LLO biosynthesis is not an obligatory result of ER stress and are consistent with the hypothesis that glucosamine-induces ER stress by disrupting LLO biosynthesis.

3.4 4-Phenylbutyric acid mitigates glucosamine-induced ER stress but cannot restore LLO homeostasis

4-Phenylbutyric acid (PBA) has been identified as a chemical chaperone that can assist in protein folding and alleviate ER stress. MEF cells were treated with 5 mM glucosamine or 4 μ g/mL tunicamycin in the presence or absence of 10 mM PBA for 18 hours and the effect on LLO biosynthesis was examined. Western blot analysis showed that PBA significantly reduced the ER stress markers GRP94, GRP78, and PDI that were up-regulated by glucosamine and tunicamycin (Figure 5A). Interestingly, although PBA reduced ER stress, FACE gels showed that PBA did not restore LLO homeostasis in either glucosamine or tunicamycin treated cells (Figure 5B). These results were

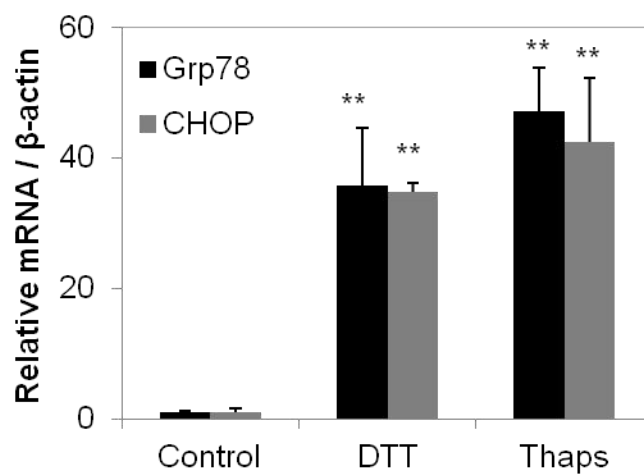
consistent with our hypothesis that glucosamine promotes ER stress by disrupting the LLO biosynthetic pathway and protein N-glycosylation. These results also highlighted the differences between glucosamine and tunicamycin induced ER stress. Although both appear to inhibit G3M9Gn2 biosynthesis, elevated concentrations of glucosamine caused an accumulation of a distinct LLO intermediate (see M5Gn2 arrow on Figure 5B).

Tunicamycin has been shown to inhibit the first enzyme in the LLO biosynthesis pathway (GPT); which subsequently inhibits the formation of all LLOs [15].

Figure 4. **DTT and Thapsigargin induce ER stress but do not disrupt LLO**

biosynthesis. MEF cells were treated with 1mM DTT or 0.5 μ M Thapsigargin (Thaps) for 4 hours. A) DTT and Thapsigargin induce ER stress and significantly elevate ER stress markers:GRP78 and GADD153/CHOP. B) FACE gel shows no change in the LLO biosynthesis under conditions of ER stress. ** $p < 0.01$; N=3.

A) RT-PCR



B) FACE gel

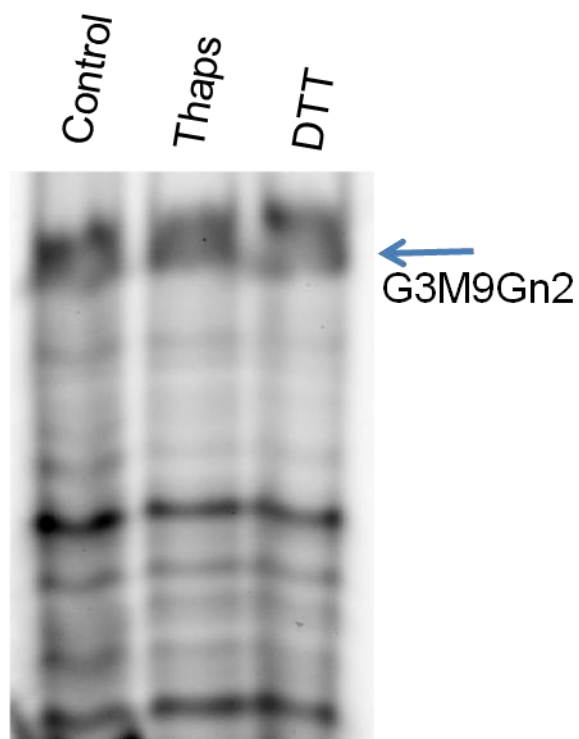
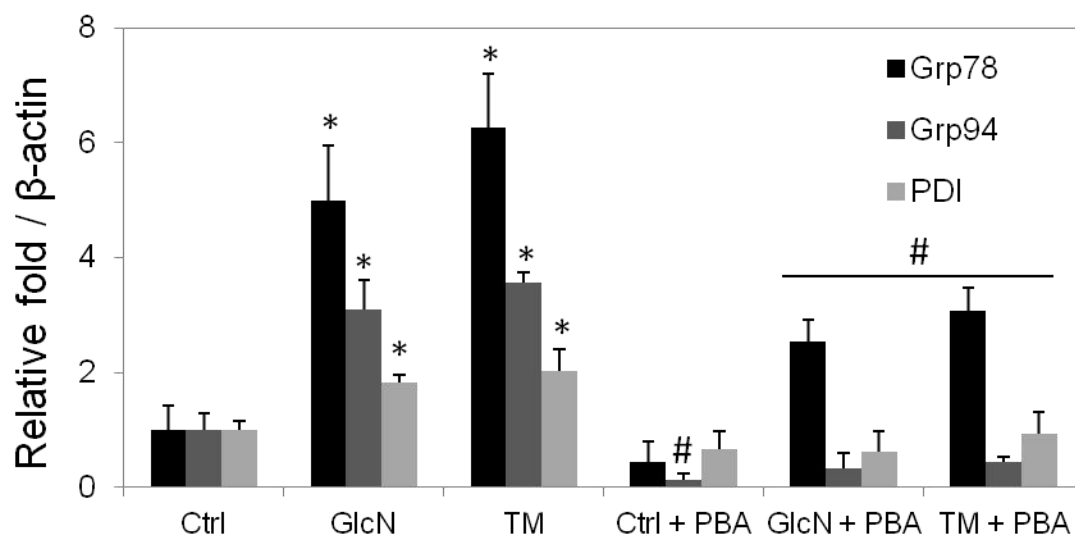
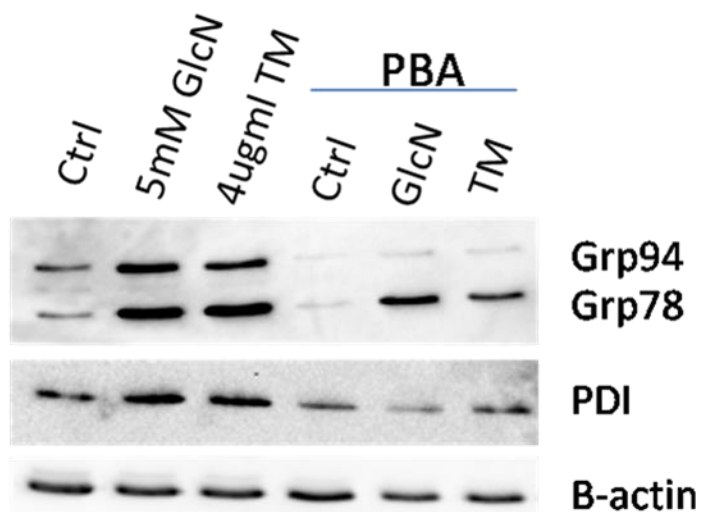
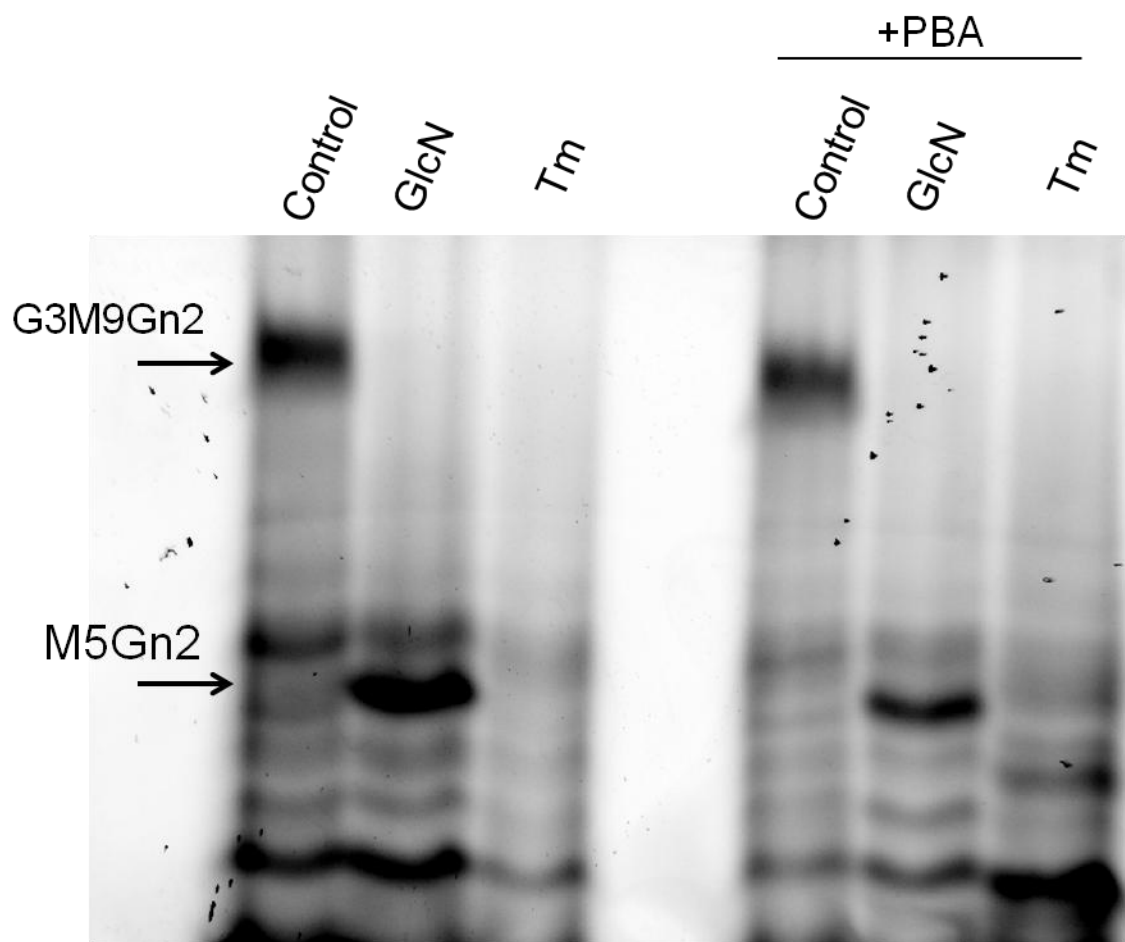


Figure 5. 4-Phenylbutyric acid mitigates glucosamine-induced ER stress but not LLO disruptions. MEF cells were treated for 18 hours with either 5 mM glucosamine (GlcN) or 4ug/mL tunicamycin (TM) in the presence or absence of 10mM 4-phenylbutyric acid (PBA). A) Cell lysates were analyzed by Western blot for ER stress markers: Grp78, Grp94 and PDI. B) FACE gels show that PBA is unable to alleviate the glucosamine and tunicamycin induced effects on the LLO pathway. * $p < 0.05$; # $p < 0.05$ relative to treatment without PBA; N=3.

A) Western blot



B) FACE gel



3.5 Glucosamine disrupts LLO biosynthesis, induces ER stress and accelerates atherosclerosis *in vivo*

It has been previously shown that mouse models of hyperglycemia (apoE^{-/-}/Ins2^{+/-akita} or streptozotocin-injected apoE^{-/-} mice) develop accelerated atherosclerosis [27-29], and that intracellular glucosamine accumulation and ER stress may play a critical role in hyperglycemia-induced atherogenesis [8, 10, 30, 31]. We have also tested this hypothesis by supplementing female apoE^{-/-} mice with 5% glucosamine (w/v) and found that it induced ER stress in atherosclerotic lesions and accelerated atherosclerosis [11]. To determine the effect glucosamine has on LLO biosynthesis *in vivo*, we supplemented female apoE^{-/-} mice with 5% (w/v) glucosamine for 10 weeks. Histological analysis showed that the glucosamine supplemented mice had significantly more O-GlcNAc, ER stress and atherosclerotic plaque area/volume compared to the controls (Figure 6A,B). There were no significant differences in fasting blood glucose levels, plasma triglyceride or total plasma cholesterol levels of glucosamine supplemented mice relative to controls (Figure 6C). FACE analysis showed that LLOs in hepatic tissue from glucosamine-supplemented mice were disrupted with an increase in LLO intermediates (see arrow in Figure 7). An increase in a similar LLO intermediate was also found in cell culture treatments with glucosamine (Figure 2,3), and these results suggest that glucosamine-induced ER stress *in vitro* and *in vivo* may be caused by disrupting the LLO biosynthetic pathway.

Figure 6. Glucosamine induce ER stress and atherogenesis in apoE^{-/-} mice.

Comparison of atherosclerotic lesion area, vascular O-GlcNAc levels, and ER stress in aortas from control or glucosamine-supplemented (GlcN-suppl) female apoE^{-/-} mice. A) Images of representative cross sections of aortic root stained as indicated. Arrows indicate representative positively stained cells. B) Quantification of atherosclerotic lesion area and immunostaining. C) Select metabolic parameters of the different models.

*p<0.05, **p<0.01 compared to control, N=8/group. FBG: fasting blood glucose; TG: triglycerides, chol: total cholesterol.

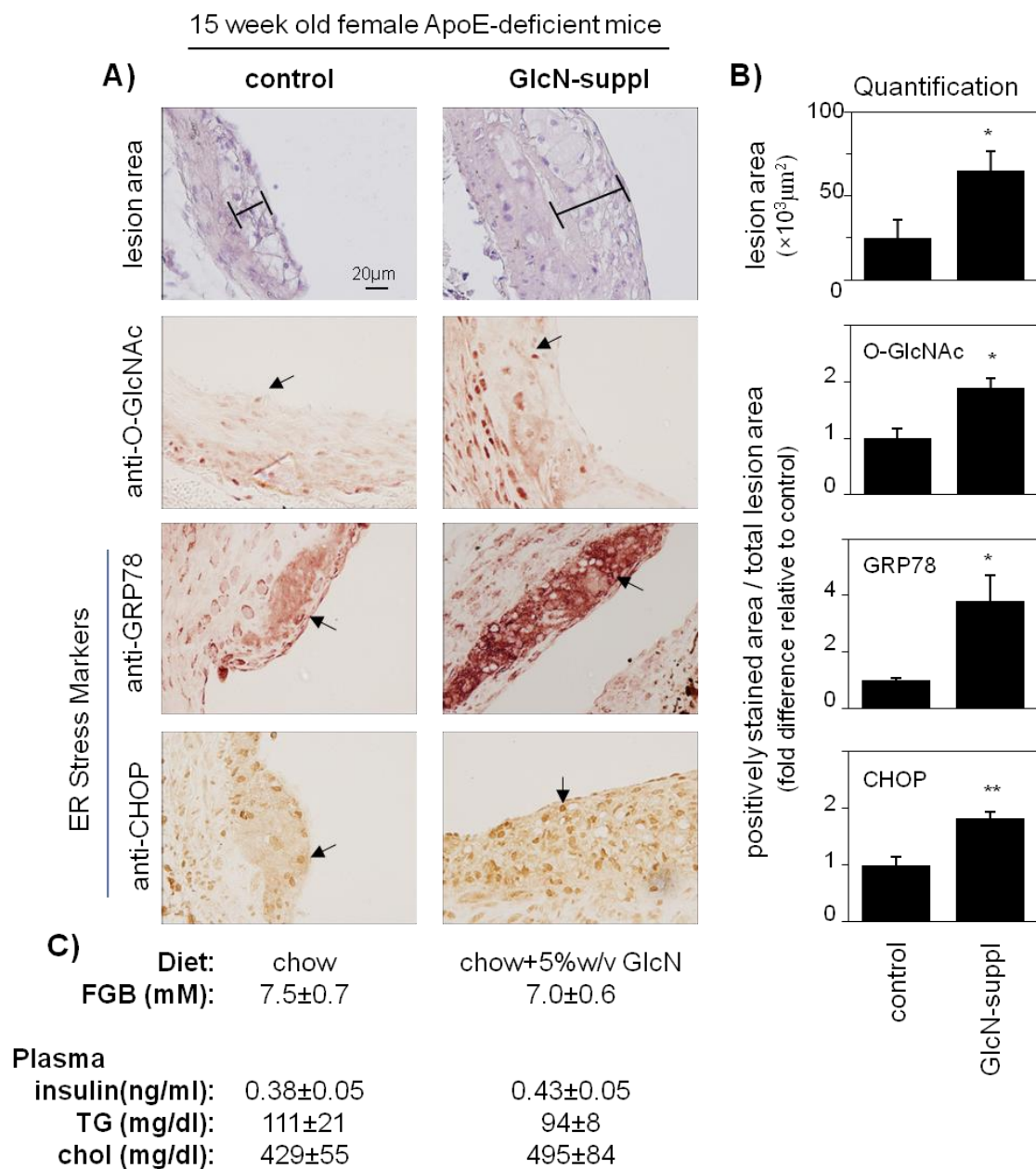
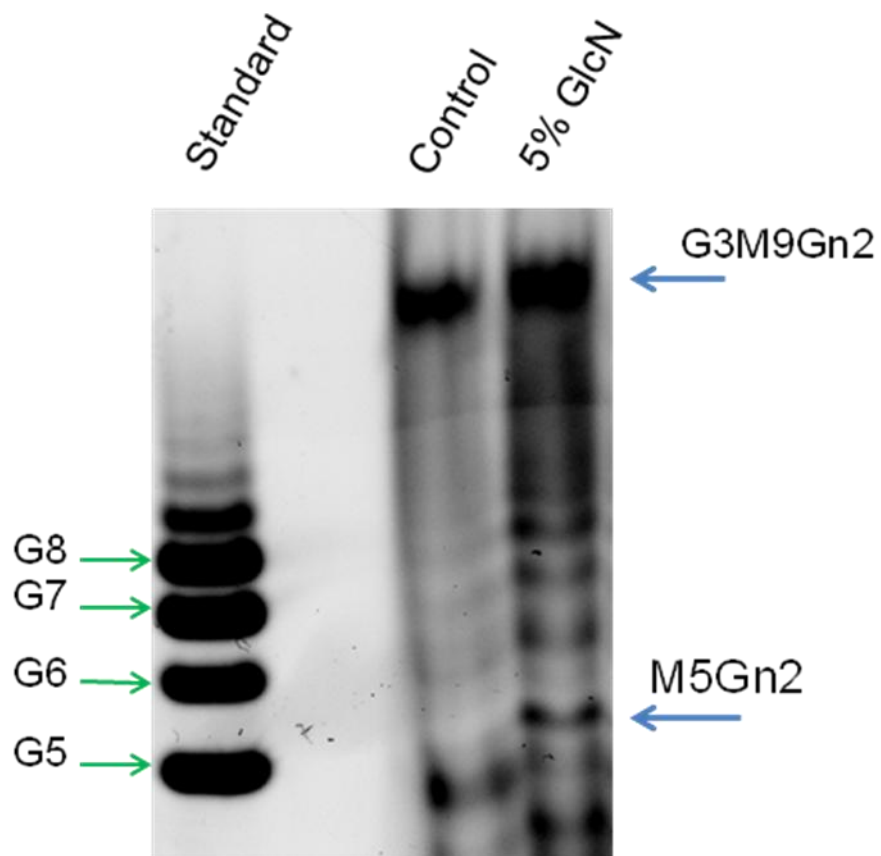


Figure 7. Glucosamine-supplementation disrupts LLO biosynthesis in apoE^{-/-} mice.

Hepatic tissue (0.5g) from apoE^{-/-} mice supplemented with or without 5% w/v glucosamine were processed and analyzed by FACE gel. The blue arrow indicates the buildup of an intermediate LLO in the glucosamine-supplemented mice. The standard in the far left lane is a glucose polymer of 5 to 8 monomers and is the same standard seen in figure 3D.



4. Discussion

Several mechanisms have been proposed to explain the pro-atherogenic effects of hyperglycemia. These include increased flux through the polyol pathway, the formation of advanced glycation end-products (AGEs), and activation of protein kinase C (PKC). Each of these mechanisms are induced/enhanced by oxidative stress and result in the activation of pro-inflammatory pathways in vascular cells [32-34]. While pre-clinical evidence supports a causative role for oxidative stress in atherogenesis [35, 36], all well-controlled clinical trials have failed to show a cardiovascular benefit in diabetic patients receiving anti-oxidant treatments [37, 38]. This suggests that oxidative stress may not be the only contributor to diabetes associated atherosclerosis.

Under physiological conditions, 1–3% of intracellular glucose is shunted from the glycolytic pathway to the HBP (see Figure 3 from thesis introduction). Flux through the HBP directly correlates with glucose concentration [39-41]. Activation of the HBP has been implicated in the development of diabetic complications including glucotoxicity [42], insulin resistance [43], and cardiomyocyte dysfunction [44], and traditionally, these effects are thought to be mediated by increasing protein O-glycosylation [39]. The O-linked glycosylation of specific serine/threonine residues is a dynamic process that is regulated by two enzymes: O-GlcNAcTransferase (OGT) and O-GlcNAcase (OGA) [39]. Increased HBP flux results in increased O-GlcNAcylation of many proteins including transcription factors (SP1, YY1, FoxO1), nuclear pore proteins (p62), as well as signaling factors (eNOS, IRS-1) [39, 40, 45]. The majority of studies implicating enhanced HBP flux to downstream pathologies focuses upon effects on O-linked glycosylation and do

not look at the effects on protein N-glycosylation. We have identified an additional consequence of enhanced HBP flux that involves the disruption of protein processing in the endoplasmic reticulum – a condition known as ER stress. Evidence from our lab and others has implicated ER stress in the activation of pro-atherosclerotic pathways [46-51].

Elevated concentrations of glucosamine increase the flux through the HBP and appear to have a counter intuitive effect on N-linked glycosylation. While glucosamine may increase O-glycosylation of specific proteins such as p62 [45], it appears to also decrease protein N-glycosylation of specific proteins such as apoB100 [13]. The multiplicity of effects glucosamine may have on protein trafficking, function, and stability, as well as its ability to cause a chronic ER stress response may have a profound effect on variety of pathologies, including atherosclerosis. Our results show that supplementing apoE^{-/-} mice with glucosamine can disrupt LLO biosynthesis, induce ER stress and accelerate atherosclerosis independent from changes in plasma cholesterol levels, triglyceride levels or glucose tolerance (Figures 6 and 7). These results suggest that the HBP may play a critical role in hyperglycemia-induced atherogenesis.

Although 4-phenylbutyric acid (PBA) is believed to act as a chemical chaperone that aids in protein folding, the specific mechanism of action has not been described in the literature. It was remarkable that PBA mitigated the ER stress response in our study, even though LLO biosynthesis and N-glycosylation was severely disrupted by glucosamine or tunicamycin (Figures 5A and B). Evidence in the literature has shown that it is possible to mitigate the ER stress response by overexpressing native protein chaperones, such as GRP78 [52], even in the presence of an ER stress stimulant. In fact,

there are previous studies that have shown PBA can alleviate ER stress-induced atherogenesis in mice fed a high fat/cholesterol diet [53, 54]. Together, these studies suggest that the downstream pathways of chronic ER stress play a larger role in diseases like atherosclerosis, beyond the effects on specific proteins. Therefore, enhancing the protein folding capacity of the cell may be a novel solution to diseases caused by chronic ER stress.

There are distinct differences between the effects of glucosamine in cell culture and in apoE^{-/-} mice. Acute treatment of glucosamine in cultured cells has a profound effect on LLO biosynthesis; our results showed a lack of G₃M₉Gn₂-P-P-Dol and a significant accumulation of a LLO intermediate (Figures 2 and 3). However, chronic exposure to 5% (w/v) glucosamine for 10 weeks appeared to induce an adaptive response in our apoE^{-/-} mice. FACE gels show that the level of G₃M₉Gn₂-P-P-Dol appears to be similar between mice supplemented with or without glucosamine. However, mice supplemented with glucosamine accumulate LLO intermediates similar to that seen in cell culture (Figure 7). These results were not unexpected as we hypothesized that a complete lack of N-glycosylation would likely be lethal to the mice. The glucosamine supplemented mice were undergoing a chronic ER stress response (Figure 6), and as such it may be possible that these cells adapted out of necessity due to the deleterious effects on N-glycosylation. In fact, the UPR has been shown to regulate a variety of enzymes involved in LLO biosynthesis including glycosyltransferases [55-57], OST [56, 57], and even enzymes in the HBP such as GFAT [58]. Adaptation by the UPR to regulate enzymes in the LLO biosynthesis pathway to allow for the generation of G₃M₉Gn₂-P-P-

Dol would require the UPR to be continuously activated. Theoretically, the UPR has been likened to a protein-folding thermostat [59] due to the self-regulated response a cell initiates to normalize disruptions to ER homeostasis. Misfolded proteins accumulate within the ER lumen, which initiates the UPR, and acts to restore protein homeostasis, which in turn, deactivates the UPR. This is thought to be a normal cyclic process in healthy cells, whereas cells stressed with agents like glucosamine would tend to be exposed to chronic ER stress and constant UPR activation.

Disruptions in protein N-glycosylation can have disastrous effects in humans and are the cause of a group of disorders known as type 1 congenital disorders of glycosylation (CDG). Twelve distinct genetic disorders have been identified that affect specific glycosyltransferases as well as other enzymes and factors in the LLO biosynthetic pathway [60-62]. Although each CDG type has a different phenotype, clinical manifestations begin in infancy and range from severe developmental delay and hypotonia with multiple organ dysfunctions to normal development with hypoglycemia and protein-losing enteropathy. Fibroblasts isolated from patients with type 1 CDG have been shown to exhibit chronic ER stress [63] however little is known regarding the pathophysiological effects of ER stress in these individuals.

In conclusion, increasing the flux through the HBP can disrupt LLO biosynthesis and subsequently induce ER stress and accelerate atherosclerosis in apoE^{-/-} mice. In cell culture, our results showed that glucosamine disruptions in LLO biosynthesis precede UPR activation and that these LLO disruptions are not a byproduct of ER stress itself. Although both glucosamine and tunicamycin disrupt LLO biosynthesis, our results

suggest they differ mechanistically as glucosamine treated cells accumulate an LLO intermediate. Interestingly, PBA can mitigate the ER stress response caused by glucosamine or tunicamycin without correcting for the disruptions in LLO biosynthesis. As the flux through the HBP directly correlates with glucose concentrations, our results highlight the importance of this pathway in hyperglycemia-induced pathologies, such as atherosclerosis.

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5 EXAMINATION OF THE USE OF THIOFLAVIN T AS A DIRECT INDICATOR OF ER STRESS IN CULTURED CELLS AND IN TISSUE SECTIONS

5.1 Rationale

The role of ER stress in the development and progression of several important diseases underlie the need for better tools to detect and quantify the accumulation of misfolded proteins. Currently, ER stress is measured indirectly by monitoring the activation or upregulation of the endogenous UPR through RT-PCR or immunoblotting techniques. Although these techniques are accurate and well established, there are disadvantages as the required tissue processing destroys the original sample, and the analysis is indirect and retrospective in nature. Thioflavin T (ThT) is a small fluorescent molecule that has been shown to bind selectively to protein aggregates [11-14]. The goal of this aim is to evaluate the use of ThT, as a tool to detect protein aggregates/ER stress levels in living cells.

5.2 Hypothesis

We hypothesize that the fluorophore, Thioflavin T, can be utilized as an indicator of ER stress because of its ability to bind to unfolded or aggregated proteins and emit enhanced fluorescence intensity.

5.3 Objectives

- 1 Test the ability of thioflavin T to detect ER stress in live cells using known ER stress agents using fluorescent microscopy,.
- 2 Determine if thioflavin T co-localizes with misfolded proteins in the ER using confocal microscopy.
- 3 Determine if thioflavin T can also detect ER stress in fresh frozen tissues from various mouse models.

Detection and quantification of endoplasmic reticulum stress in living cells using the fluorescent compound, Thioflavin T

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*Running Title: *Detection of ER stress in living cells*

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Abstract

The endoplasmic reticulum (ER) plays a central role in the co- and post-translational modification of many proteins. Disruption of these processes can lead to the accumulation of misfolded proteins in the ER – a condition known as ER stress. In recent years, the association of ER stress with a number of diseases pathologies has increased interest in the study of this condition. Current methods to detect ER stress are indirect and retrospective. Here we describe a new method to detect and quantify ER stress in live cells using Thioflavin T (ThT), a small molecule that exhibits enhanced fluorescence when it binds to protein aggregates. We show that enhanced ThT-fluorescence correlates directly with established indicators of unfolded protein response (UPR) activation. Furthermore, enhanced ThT-fluorescence can be detected in living cells within 20 minutes of application of an ER stress-inducing agent. ThT is capable of detecting ER stress induced by distinctly different conditions and compounds, in different cultured cell types as well as in mouse tissue samples. Pre-treatment with a potent ER stress-reducing agent, 4-phenylbutyric acid, mitigates the enhanced ThT signal. This new tool will be useful in future research investigating the role of protein misfolding in the development and/or progression of human diseases.

Keywords: thioflavin T, endoplasmic reticulum stress, unfolded protein response, detection

1. Introduction

The endoplasmic reticulum (ER) plays a central role in lipid biosynthesis and maintenance of calcium homeostasis. In addition, the ER is responsible for the efficient trafficking of approximately one third of all proteins produced in a typical eukaryotic cell. Central to this function is the co- and post-translational processing of proteins, including disulfide bond formation and N-linked glycosylation, which are essential for proper protein folding [1]. Under conditions where the influx of nascent proteins exceeds the processing capacity of the ER, unfolded or misfolded proteins can accumulate and disrupt ER homeostasis – a condition known as ER stress. Conditions of ER stress activate the unfolded protein response (UPR), which is a cellular self-defense mechanism that functions to alleviate ER stress by decreasing protein synthesis, enhancing protein folding capacity, and increasing the degradation of irreversibly misfolded proteins [2,3].

ER stress and/or activation of the UPR has been implicated in the development of several human pathologies, including neurodegenerative disorders (Alzheimer, Parkinson), diabetes mellitus, obesity, cancer and cardiovascular disease [4,5]. Therefore it is possible that factors that are indicative of ER stress and UPR activation could act as diagnostic markers of disease development or progression. Furthermore, if ER stress plays a causative role in disease pathogenesis, then this pathway may contain targets for therapeutic intervention.

ER stress can be measured indirectly by monitoring the activation/upregulation of various components of the endogenous UPR. Typically this involves using qRT-PCR or immunoblotting techniques to detect changes in UPR mRNA and protein levels,

respectively [6]. The advantage of this strategy is that the methods are well established, the required molecular tools are readily available, and when performed and analyzed correctly, the results are accurate and consistent indicators of ER stress. However, these procedures are retrospective in nature because the ER stress markers are quantified hours after the cells are harvested. In addition, these methods indirectly assess ER stress, through the measurement of specific components of the UPR. Finally, multiple UPR genes products have to be monitored because other, ER stress-independent, pathways can affect expression of individual UPR genes (see review [6]).

Alternative systems have been developed to detect UPR activation using exogenous sensors that are inserted into the cells of interest. These include the secreted alkaline phosphatase (SEAP), XBP-1-venus fusion constructs, and reporter constructs containing XBP-1 or ATF6 binding sites [7-10]. The SEAP and XBP-1-venus systems have been shown to work *in vivo* as well as in cultured cells. The limitation of all of these detection systems is that they require the introduction of an exogenous transgene reporter.

The growing appreciation of the role of ER stress in the development and progression of several important diseases together with the limitations of the currently available systems of ER stress/UPR detection underlie the need for a more versatile technique to rapidly detect and quantify misfolded proteins. Thioflavin T (ThT) is a small molecule with fluorescence properties that has been shown to bind selectively to protein aggregates, particularly β -sheets [11-14]. The objective of this study is to characterize

and evaluate the effectiveness of ThT, as a tool to detect protein aggregates as a measure of ER stress levels in living cells.

2. Methods

2.1 Cell culture

Mouse embryonic fibroblasts (MEFs) and human hepatocarcinoma (HepG2) cells were cultured in 1x DMEM (Invitrogen, Burlington, ON) containing 10% FBS (Hyclone, Rockford, IL). Human aortic endothelial cells (HAECs) were cultured in medium 200 (Cascade Biologics, Portland, OR) containing 10% Low Serum Growth Supplement (Cascade Biologics). All cells were maintained at 37°C in 5% CO₂. Cells were split with 1x trypsin into 6-well culture dishes and allowed to adhere overnight before incubation with specific treatments for 0-18 hours. Cells were washed with 1x PBS and treatments were filter sterilized and diluted in culture media prior to application. ThT (Sigma, St. Louis, MO) was dissolved in 0.5 mL 70% ethanol and diluted in 5.5 mL media to create a 5 mM stock solution. The stock solution was further diluted in media to a final concentration of 5 µM. Glucosamine hydrochloride (Sigma) and 4-phenylbutyric acid (4PBA) (Science Lab, Houston TX) were diluted in media and adjusted to pH 7.2. DTT (Sigma) and thapsigargin (Sigma) were diluted in media, and palmitate (Sigma) was prepared in BSA and media as previously described [15].

2.2 Immunoblot analysis

Monoclonal antibodies to Grp78/94 (anti-KDEL, Stressgen Biotechnologies, Victoria, BC), GADD153/CHOP (Santa Cruz, Santa Cruz, CA), and anti-β-actin (Sigma-

Aldrich) were used for protein detection/quantification by immunoblot analysis. Total protein lysates from cell culture were normalized by Bradford assay, solubilized in 4x SDS-PAGE sample buffer and separated on SDS-polyacrylamide gels under reducing conditions. Blots were incubated with the appropriate primary antibody for 18 hours at 4°C and subsequently with an anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO, Burlington, ON). Blots were developed using the Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA), and visualized with the ChemiDoc XRS System (BioRad). Band densities were quantified using Image Lab software.

2.3 Fluorescence detection and quantification

Fluorescent images of live cells were taken at a 500 ms exposure under a 10x objective (N.A. 0.25) with an Olympus CKX41 microscope (Ex. BP460-490 nm, barrier Em. 520 nm) and Infinity1-3C camera and saved using Infinity Analyze software (Lumenera Corp., Ottawa, ON). Fluorescent images were converted to 8-bit before being quantified by threshold analysis on ImageJ. Cells from each image were manually counted to normalize the fluorescent data per cell. Signal intensity, and assay sensitivity, can be increased by using objectives with a higher numerical aperture. Confocal images of 4% paraformaldehyde/PBS fixed cells were taken with a 63x glycerol immersion lens on a Multiphoton Leica TCS SP5 confocal microscope (Dr. Ray Truant's Lab, McMaster University). The excitation and emission settings were: DAPI (Ex. MP laser 800 nm, Em. 410-530 nm), ThT (Ex. 458 nm, Em. 480-520 nm), and Alexa568 (Ex. 561 nm, Em. 575-645 nm). Each fluorescent channel was imaged sequential, as opposed to simultaneously,

to avoid channel overlap. Cells were fixed for 20 minutes at room temperature, briefly permeabilized (5-30 sec) with 0.05% tritonX/PBS, blocked for 20 minutes with 5% BSA/PBS and incubated with 1:700 mouse anti-KDEL antibody/PBS overnight at 4°C. Cells were PBS washed (3x), then incubated with 1:700 Alexa568 anti-mouse secondary antibody/PBS for 1 hour at room temperature (protect from light). Cells were PBS washed (3x), then incubated with RTU DAPI for 1 minute, and washed with dH2O (3x). Cells were incubated with 5 µM ThT for 10 minutes (do not wash), and mounted to slides using 2 drops of Fluoromount and store at 4°C in the dark.

2.4 Mouse tissue

Fresh frozen apoE^{-/-} mouse liver was sectioned, collected onto cover slides and stored at -20°C. Prior to ThT staining, sections were allowed to thaw for 5 minutes and then fixed in 37% formaldehyde for 2 minutes at room temperature. Sections were then washed 2× with water, and incubated with fresh filtered 500 µM ThT in PBS for 3 minutes at room temperature. Slides were washed in water for 3 minutes. To preserve the fluorescence, slides were coated with 2 drops of Fluormount (Sigma) and cover-slips were applied before imaging. The McMaster University Animal Research Ethics Board approved of all procedures.

2.5 Statistical analysis

Data are expressed as mean ± standard deviation. A significance of differences was determined using an unpaired t-test of equal variance. *P* values < 0.05 are considered statistically significant.

3. Results

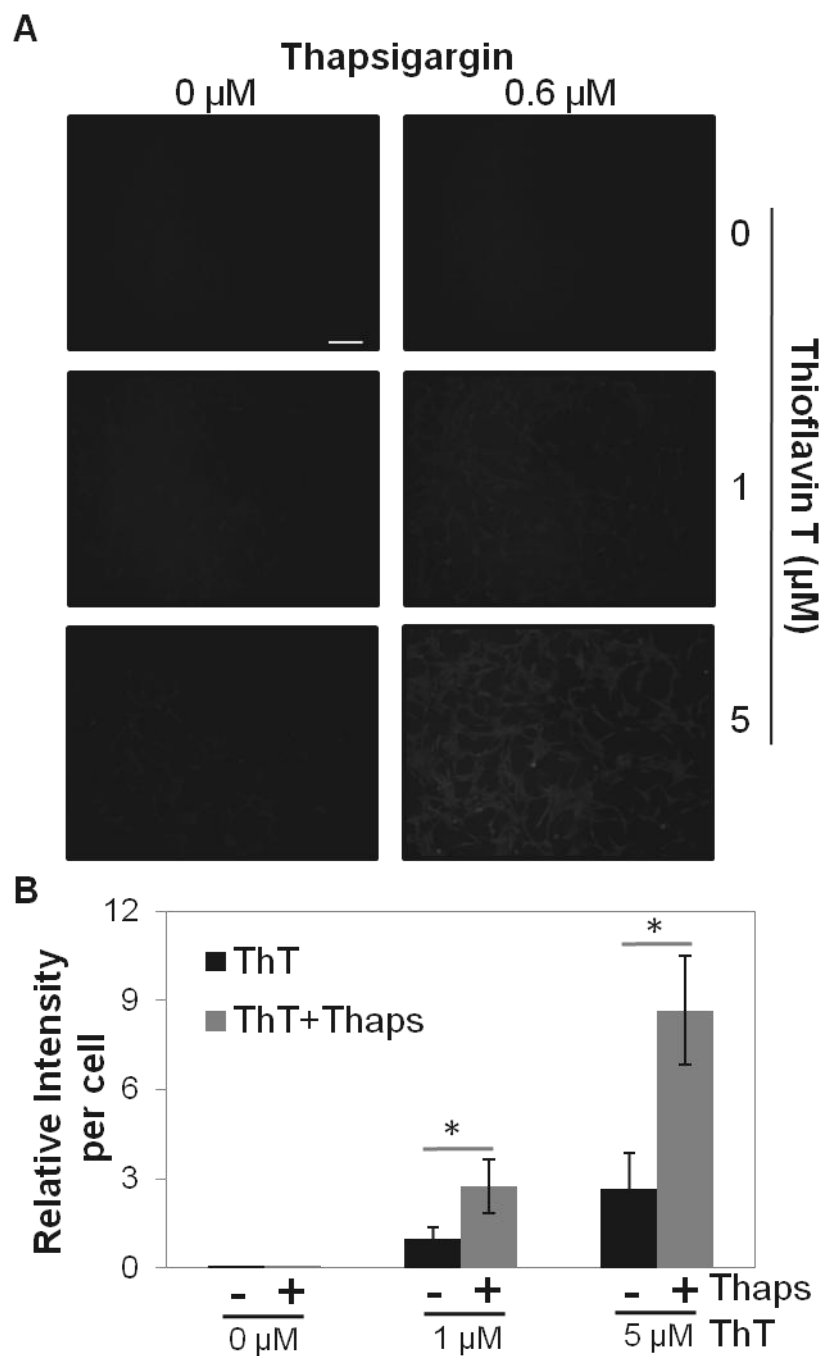
3.1 The effect of ER stress on ThT fluorescence intensity

Our first aim was to determine the effect of ER stress on ThT fluorescence in living cells and also to identify the concentration of ThT that is required to give a significant and detectable signal. Mouse embryonic fibroblasts (MEFs) were cultured in the absence (0 μM) or the presence (1 μM) of the ER stress-inducing agent, thapsigargin [16], for 18 hours. Increasing concentrations of ThT (0-50 μM) were added to the cultures. Low level background fluorescence (auto-fluorescence) was detected in the absence of ThT. In unstressed cells (0 μM thapsigargin), a linear increase in fluorescence was detectable with increasing ThT concentration (Figure 1). Relative to controls, fluorescence was significantly enhanced in thapsigargin treated cells. The maximum differential fluorescence (5.5 fold, $P < 0.01$) was observed in the presence of 5 μM ThT. Concentrations ≥ 50 μM ThT were toxic, and resulted in substantial cell death (data not shown). In all subsequent experiments, ThT was used at a concentration of 5 μM .

To determine if the intensity of fluorescence directly correlated to the level of UPR activation, MEFs were cultured in the presence of increasing concentrations of thapsigargin (0-1 μM) for up to 18 hours. ThT (5 μM) was added to all cells. Images of living cells were captured, and fluorescence was quantified (Figure 2A). The same cells were then harvested, proteins were resolved by SDS PAGE, and blots were probed with antibodies against established markers of ER stress, Grp78 and GADD153/CHOP (Figure 2B). As expected, increasing the concentration of thapsigargin resulted in increased Grp78 and GADD153/CHOP protein levels. A similar, linear increase in ThT

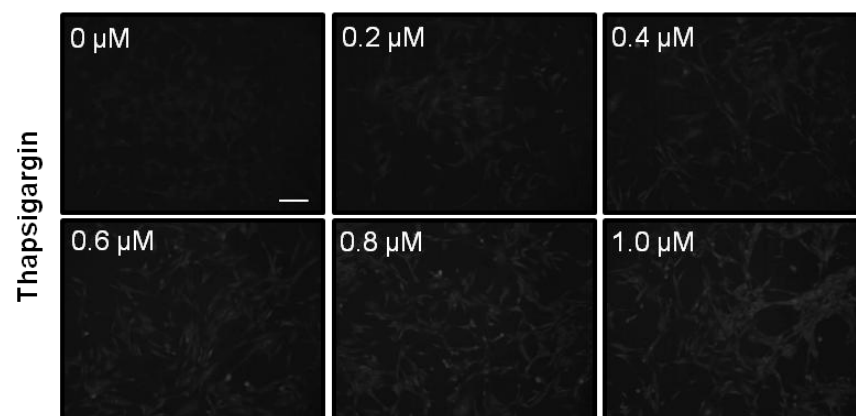
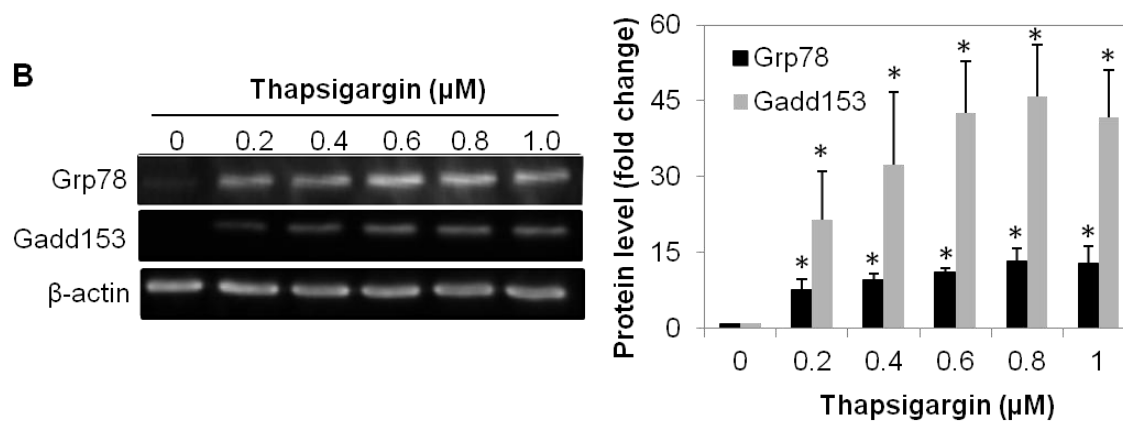
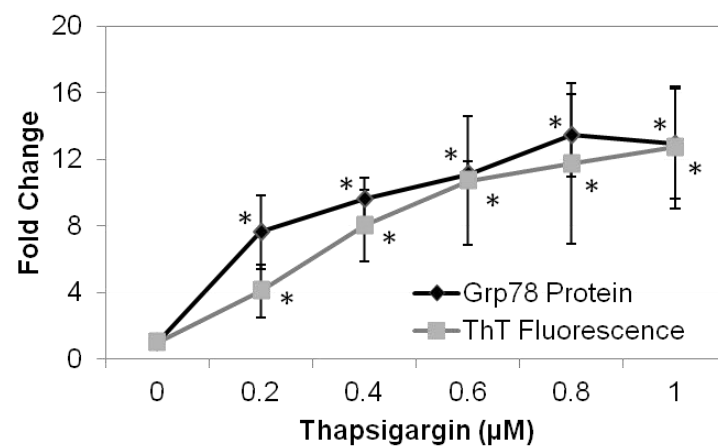
fluorescence intensity was also observed in these cells. This result suggests that there is a direct correlation between ThT fluorescence intensity and UPR activation in living cells (Figure 2C).

Figure 1. **Increased ThT fluorescence in the presence of thapsigargin.** Mouse embryonic fibroblasts (MEFs) were treated with 0 or 1 μ M thapsigargin (Thaps) and 0, 1 or 5 μ M ThT for 18 hours. **A)** Live cell images were captured and **B)** fluorescence intensity was determined and quantified. A significant increase in fluorescence was observed with 5 μ M ThT. * $P < 0.05$, $n = 3$, Scale bar = 100 μ m



Beriault, D. and Werstuck, G. BBA Molecular Cell Research 2013

Figure 2. ThT fluorescence intensity corresponds to ER stress-induced activation of the unfolded protein response. MEFs were treated with 0-1.0 μ M thapsigargin and 5 μ M ThT for 12 hours. **A)** Live cell images were captured and fluorescence intensities were determined. **B)** Cells were harvested and total protein lysates were resolved for immunoblot analysis using antibodies specific for UPR proteins, Grp78 and GADD153/CHOP. **C)** The observed increase in Grp78 protein levels correlates directly with the measured increase in ThT fluorescence. * $P < 0.05$, $n=3$, Scale bar = 100 μ m

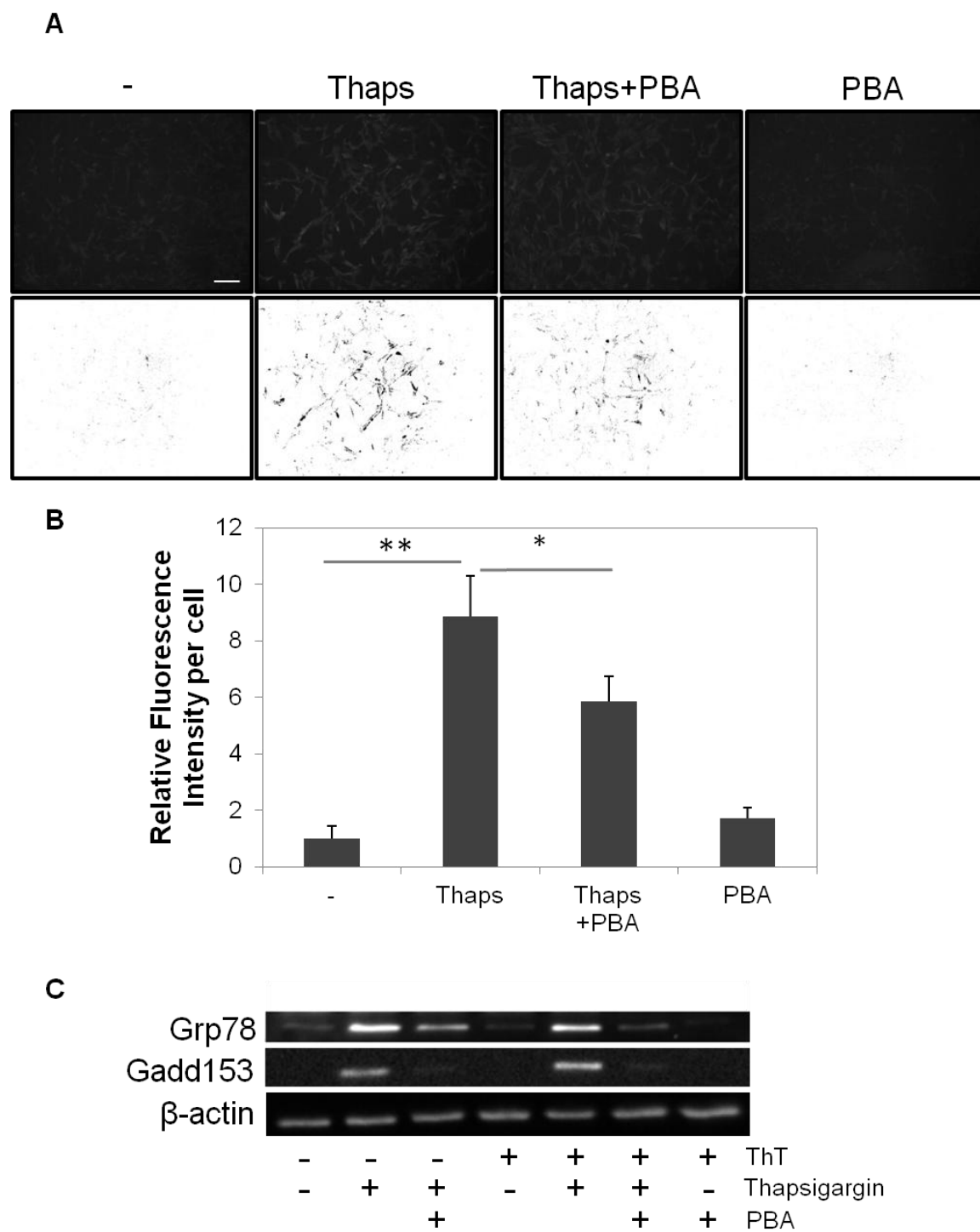
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3.2 The effects of chemical chaperones on ThT Fluorescence

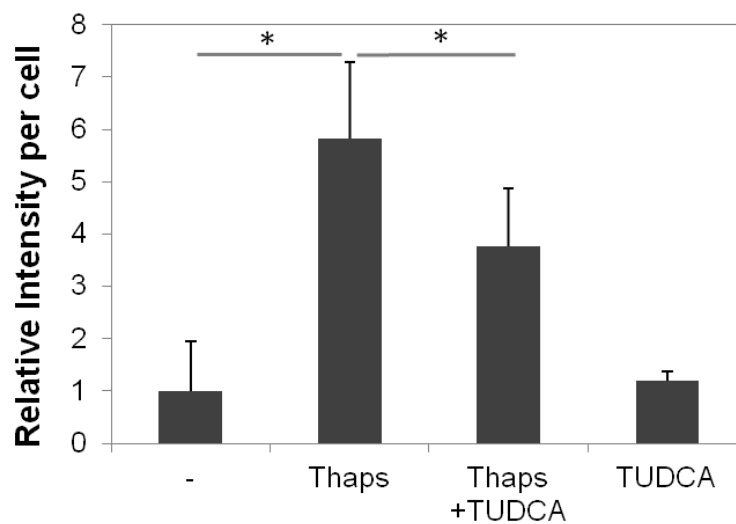
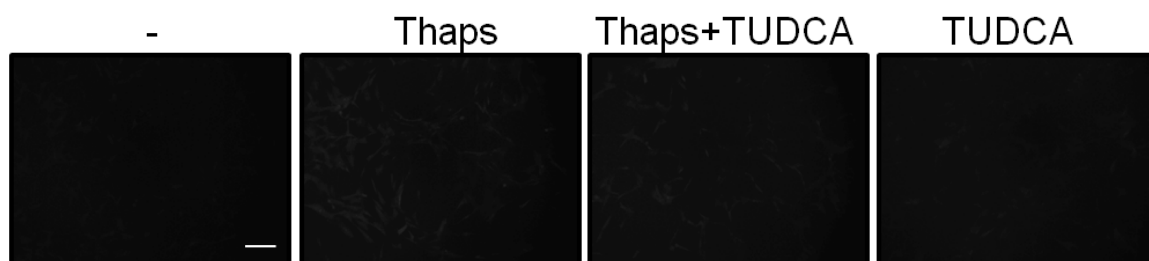
The observed direct correlation between ThT-dependent fluorescence and UPR activation is consistent with the possibility that ThT is interacting with accumulating misfolded proteins in the ER. To examine this possibility more closely, we treated cells with 0.6 μ M thapsigargin in the presence or absence of 10mM 4-phenylbutyric acid (4PBA), a chemical chaperone that has been shown to directly alleviate ER stress [17]. ThT and/or 4PBA treatment had no significant effect on fluorescence intensity (Figure 3A,B) or the expression of UPR genes (Figure 3C) in unstressed cells. Treatment with 4PBA significantly attenuated thapsigargin-induced Grp78 and GADD153/CHOP expression levels, and was also associated with a significant decrease in ThT fluorescence. Similar results were observed when ER stress was attenuated using 400 μ M tauroursodeoxycholic acid (TUDCA) (Supplementary Figure 1). These data are consistent with ThT fluorescence acting as a direct indicator of misfolded proteins.

Figure 3. **Exposure to the chemical chaperone, 4PBA, reduces UPR activation and ThT fluorescence.** MEFs were treated with 0 or 0.6 μ M thapsigargin and 5 μ M ThT as indicated for 12 hours. A subset of cells was also treated with the chemical chaperone 4PBA (10 mM). **A,B)** Live cell fluorescent images were captured (top panel), processed by ImageJ thresholding to control for background fluorescence (bottom panel), and quantified. **C)** Total protein lysates were resolved by SDS-PAGE and analyzed by immunoblot using antibodies specific for Grp78, GADD153/CHOP or β -actin, as a loading control. * $P < 0.05$, ** $P < 0.01$, $n = 3$, Scale bar = 100 μ m



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Supplemental Figure 1. **Exposure to the chemical chaperone, TUDCA, reduces ThT fluorescence.** MEFs were treated with 0 or 0.6 μM thapsigargin and 5 μM ThT for 12 hours. A subset of cells was also treated with the chemical chaperone, TUDCA (400 μM). Live cell fluorescent images were captured and quantified. * $P < 0.05$, $n = 3$, Scale bar = 100 μm



Beriault, D. and Werstuck, G. BBA Molecular Cell Research 2013

3.3 Enhanced ThT fluorescence co-localizes to the ER in stressed cells

In order to determine the subcellular localization of the ThT staining, MEFs were treated with 0 or 1.0 μ M thapsigargin for 8 hours and then stained, as described above, with ThT. The cells were subsequently probed with an anti-KDEL antibody that is specific for the ER resident chaperones Grp78 and Grp94. Multiphoton fluorescent confocal microscopy was used to visualize the staining (Figure 4) and appropriate controls (Supplementary Figure 2). As expected, there is an increase in anti-KDEL staining in the perinuclear region of thapsigargin treated cells that is consistent with increased Grp78/94 protein concentration in the ER. Enhanced ThT-fluorescence in thapsigargin treated cells colocalizes to the ER. This result is consistent with ThT interacting directly with misfolded proteins in the ER.

3.4 The ability of different ER stress-inducing agents to enhance ThT fluorescence

Thapsigargin promotes ER stress by disrupting ER Ca^{2+} homeostasis. ER stress can also be induced by disrupting disulphide bond formation (DTT) [18], disrupting N-linked glycosylation (glucosamine) [19], or by altering the composition of the ER membrane (palmitate) [20]. To determine if enhanced ThT-dependent fluorescence was indicative of ER stress induced by distinctly different compounds, MEFs were treated with 2 mM DTT, 5 mM glucosamine, or 700 μ M palmitate in the presence or absence of 10mM 4PBA. Fluorescence was quantified and UPR protein markers analyzed. Results clearly show that each of the ER stress-inducing agents tested; i) promotes expression of Grp78 and GADD153/CHOP, and ii) enhances ThT fluorescence (Figure 5 A-C). Grp78 and GADD153/CHOP expression, and enhanced fluorescence, is attenuated, in each case,

with the addition of the chemical chaperone 4PBA. This result suggests that ThT is a general indicator of ER stress.

3.5 The time course of enhanced ThT fluorescence

Indirect quantification of ER stress by immunoblot analysis of stress response proteins can only be performed after incubation times of 6 to 8 hours because of the time required to induce changes in UPR protein levels through the activation of UPR gene expression [6]. To determine how soon, after application of an ER stress-inducing agent, that an increase in ThT fluorescence is detectable, MEF were treated with ThT and 0.6 μ M thapsigargin for 0-12 hours. Images of live cells were captured and fluorescence intensity was measured. Results indicate that a significant increase in fluorescence, indicative of the accumulation of misfolded proteins, is detectable by 20 minutes after treatment with the ER stress-inducing agent (Figure 6). This time corresponds to the phosphorylation of eIF2 α , one of the earliest indicators of the activation of the UPR/PERK pathway [21,22].

3.6 The ability of ThT to detect ER stress in different cell types

The ability of ThT to detect ER stress in non-MEF cell types was investigated. A hepatocarcinoma cell line (HepG2) and primary human aortic endothelial cells (HAEC) were challenged with 1 μ M thapsigargin, 2mM DTT or 5mM glucosamine for 12 hours. ThT (5 μ M) was added to each cell culture, images were captured and fluorescence intensities quantified (Figure 7). A significant increase in fluorescence was observed in cells treated with the ER stress inducing agents. This suggests that ThT can detect ER stress in multiple cell types.

Figure 4. Co-localization of ThT and KDEL in the ER of thapsigargin-treated MEFs. MEFs were treated with 0 or 1.0 μ M thapsigargin for 8 hours before visualization. Cells were fixed, probed with an anti-KDEL antibody, and stained with DAPI and 5 μ M ThT, as indicated. Confocal images were captured using a Multiphoton Leica TCS SP5 confocal microscope. n=4, Scale bar = 10 μ m

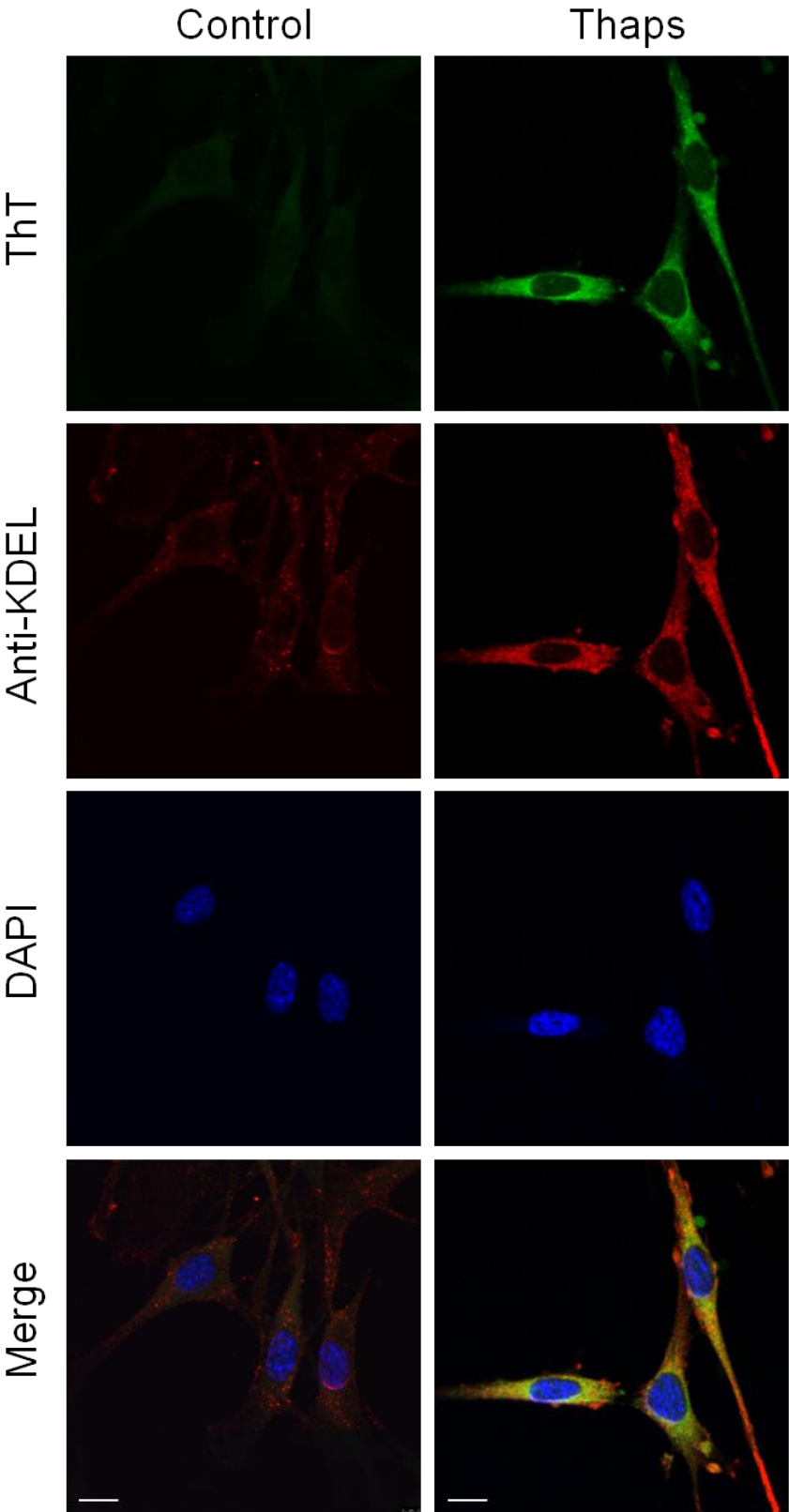
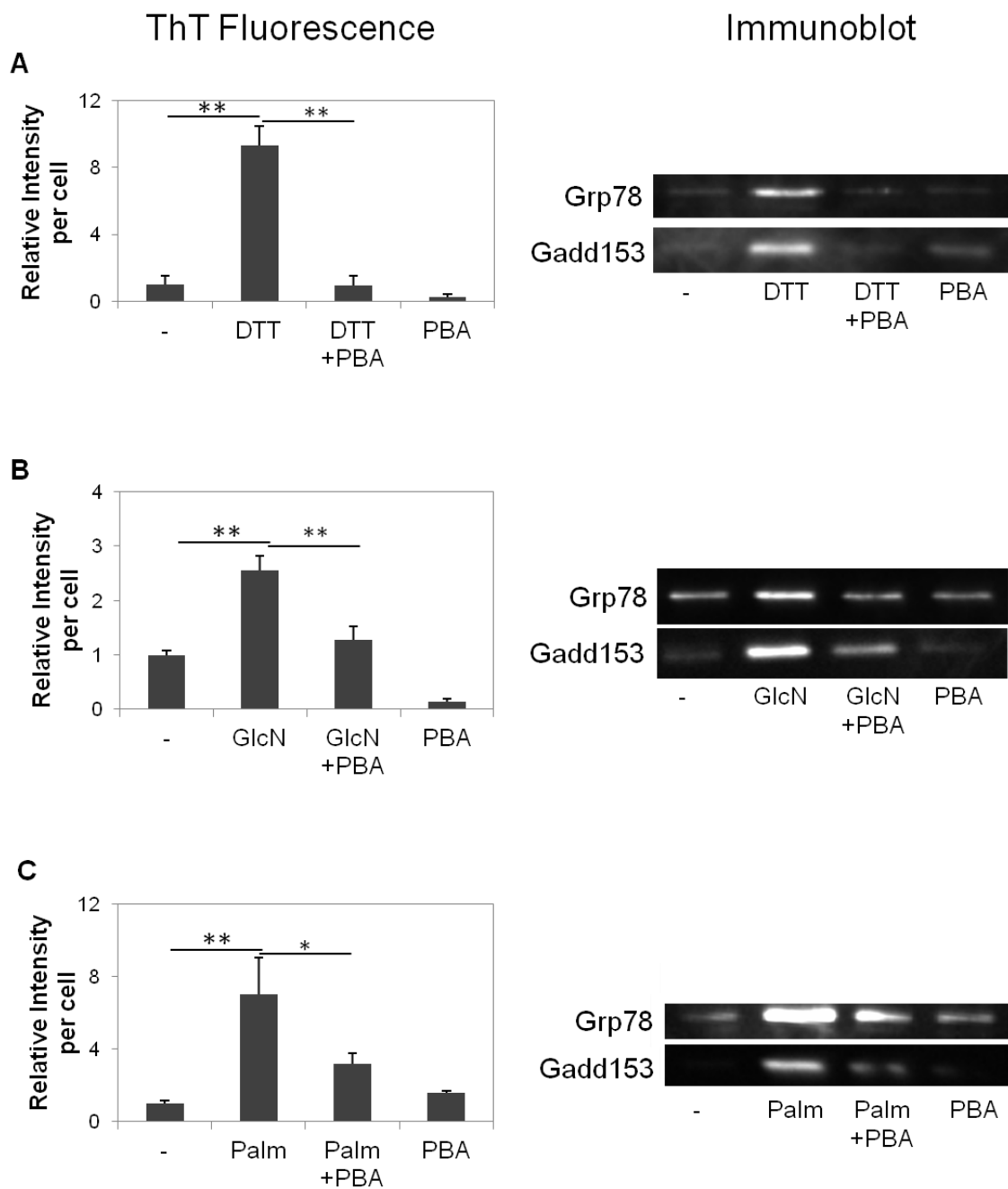


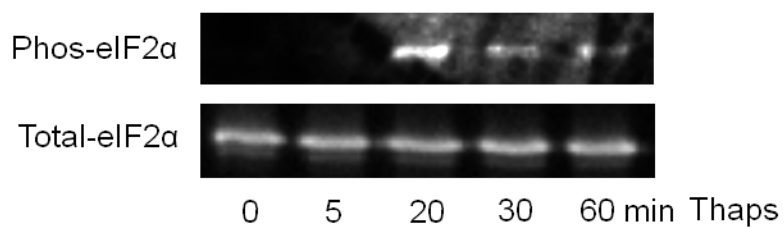
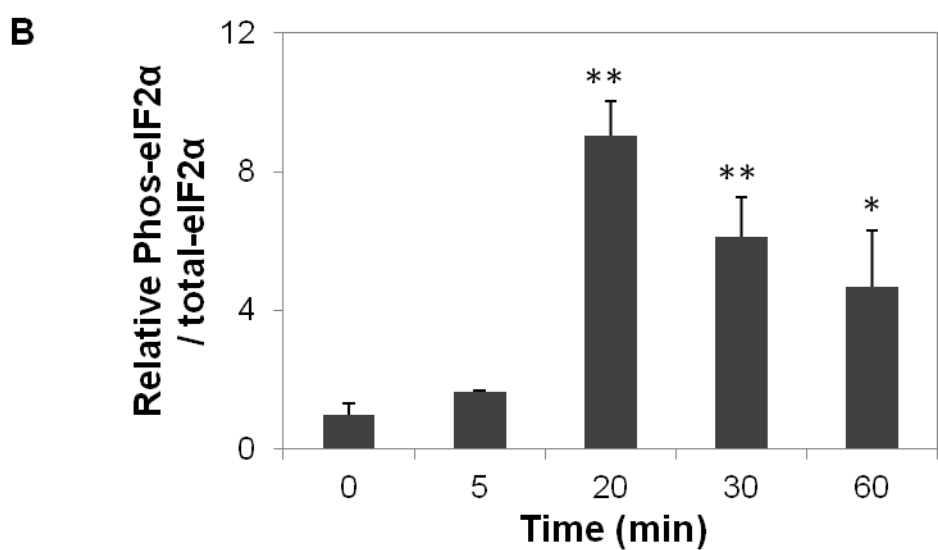
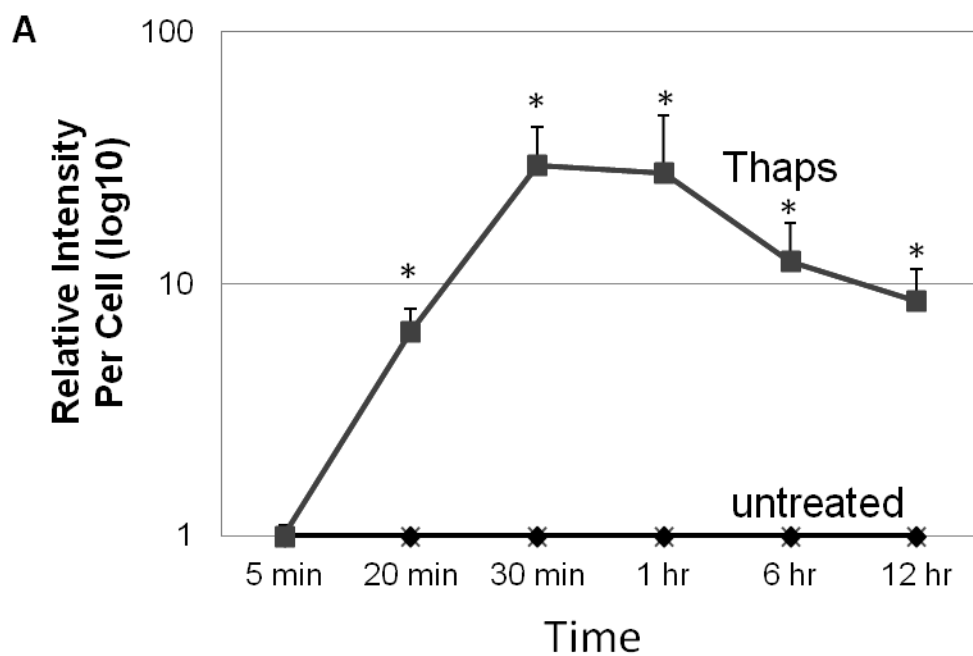
Figure 5. Exposure to different ER stress-inducing agents enhances ThT

fluorescence. MEFs were challenged with **A)** 2 mM DTT, **B)** 5 mM glucosamine (GlcN), or **C)** 700 μ M palmitate (Palm), in the presence or absence of 10 mM PBA for 12 hours, as indicated. ThT (5 μ M) was added to all cells. Live cell fluorescent images were captured and quantified and total protein lysates were immunoblotted with antibodies against Grp78 or GADD153/CHOP, as indicated. * $P < 0.05$, ** $P < 0.01$, $n = 3$



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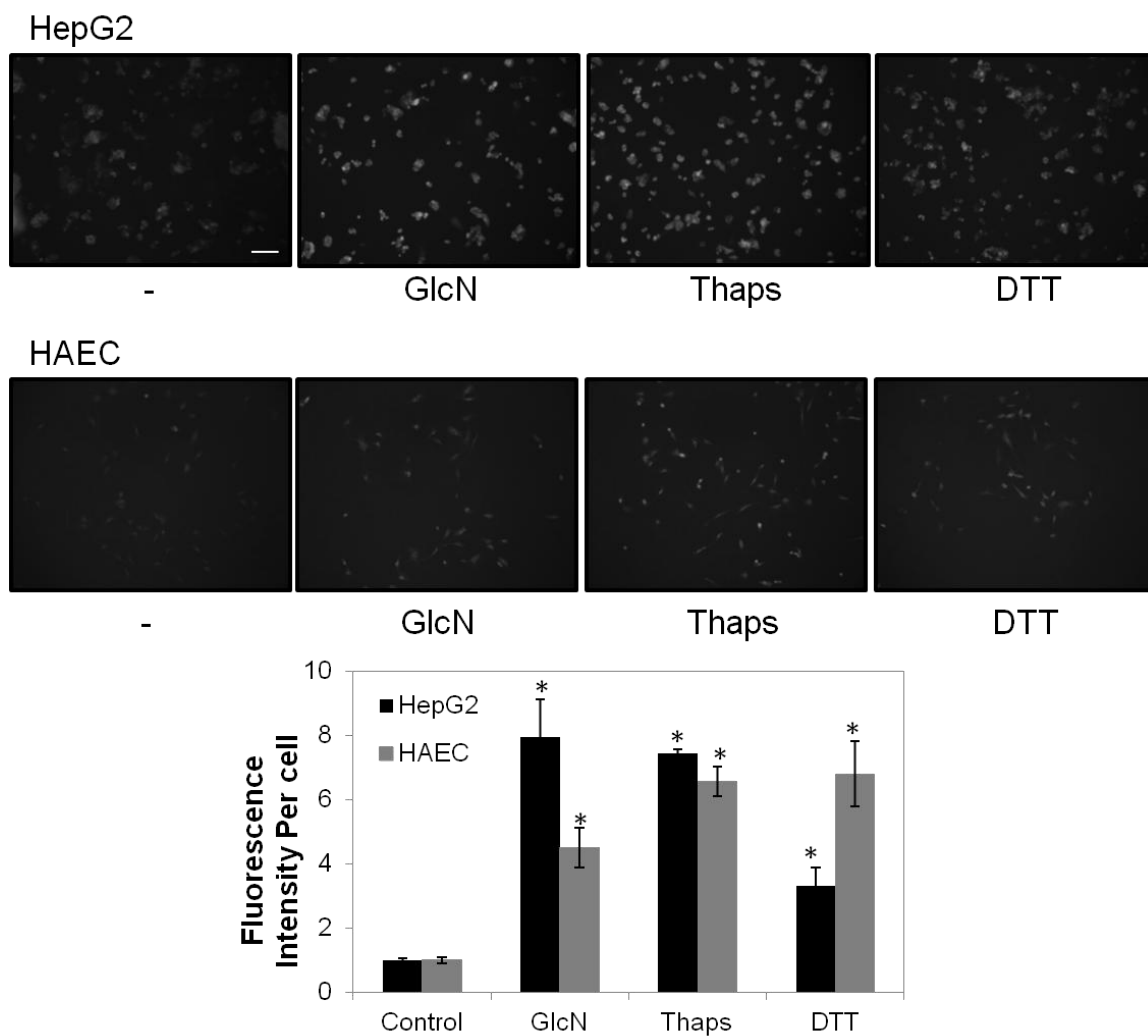
Figure 6. **Enhanced ThT fluorescence is detectable within 60 minutes.** MEFs were challenged with 0.6 μ M thapsigargin (Thaps) in the presence of 5 μ M ThT. **A)** Live cell sequential images were captured and fluorescence intensity quantified 5, 20, 30, 60, 360 and 720 minutes after the addition of the ER stress inducing agent. **B)** MEF cells were harvested at the corresponding time points for immunoblot analysis of total and phospho-eIF2 α . *P<0.05 , **P<0.01, n=3



Beriault, D. and Werstuck, G. BBA Molecular Cell Research 2013

Figure 7. ER stress promotes enhanced ThT fluorescence in different cell types.

Human hepatocellular carcinoma cells (HepG2) and Human aortic endothelial cells (HAEC) were exposed to 5 mM glucosamine (GlcN), 0.6 μ M thapsigargin (Thaps) or 2 mM DTT as indicated and live cells were imaged after 6 hours and quantified. * $P < 0.05$, $n = 3$, Scale bar = 100 μ m

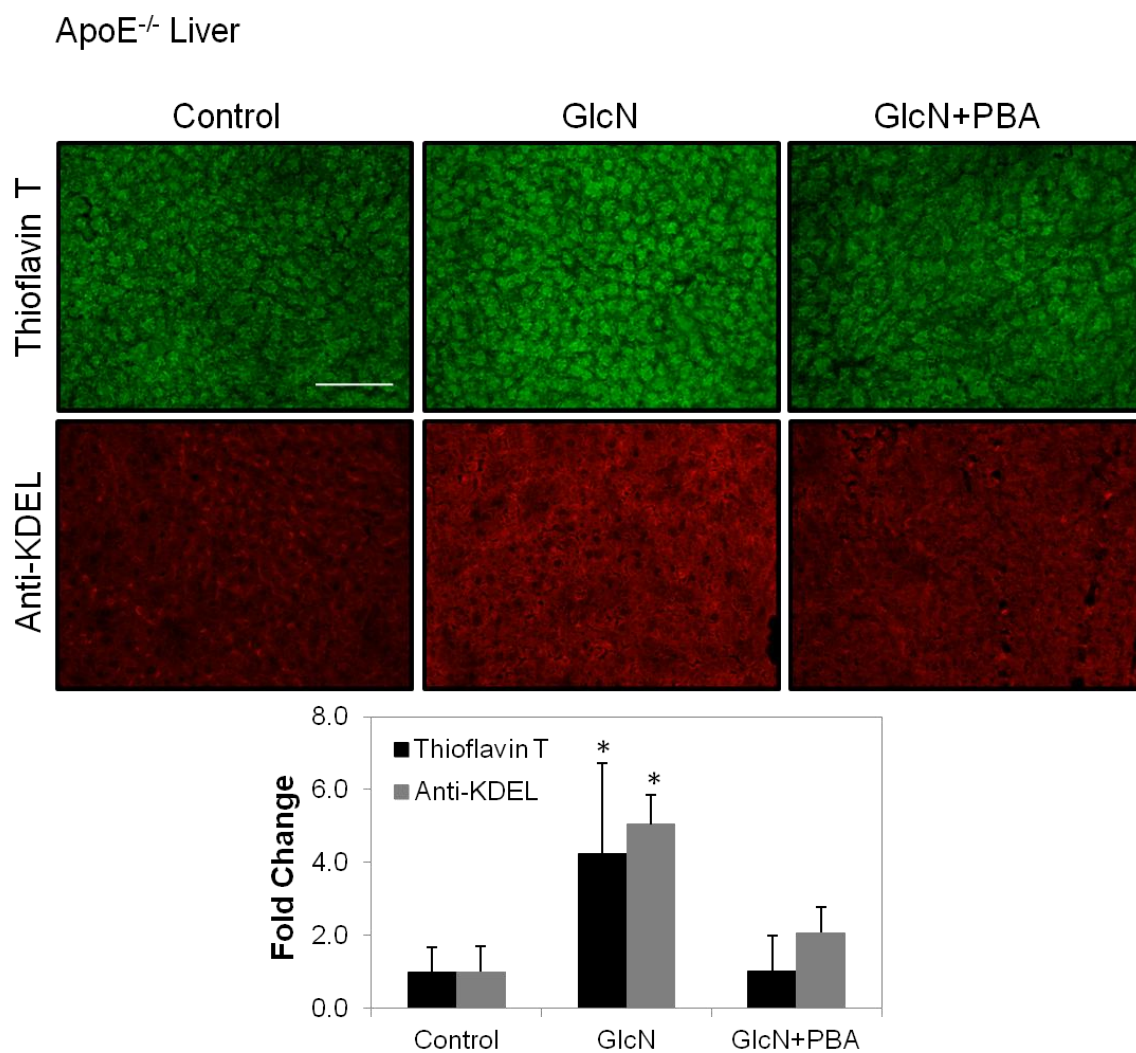


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3.7 Detecting ER stress in mouse tissue

ThT has been used extensively to detect amyloid proteins in brain tissue [11,23]. To determine if ThT could be used to detect ER stress in tissues, we examined murine liver sections. We have previously shown that streptozotocin-induced hyperglycemia [24] or glucosamine-supplementation [25] can induce hepatic ER stress in apoE^{-/-} mice. In this experiment, frozen liver sections from glucosamine-supplemented mice were stained with ThT and compared to age and gender matched control mice. The results show significantly more ThT fluorescence in glucosamine-supplemented liver relative to the controls (Figure 8). As observed in the cultured cell systems, inclusion of the chemical chaperone 4PBA [26] reduced the level of ThT fluorescence.

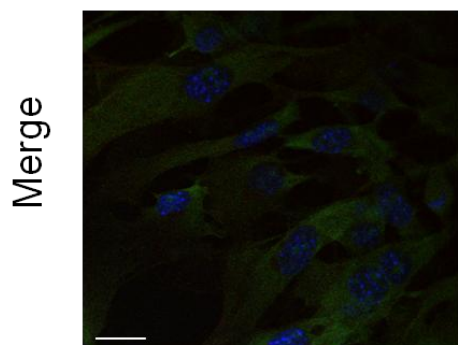
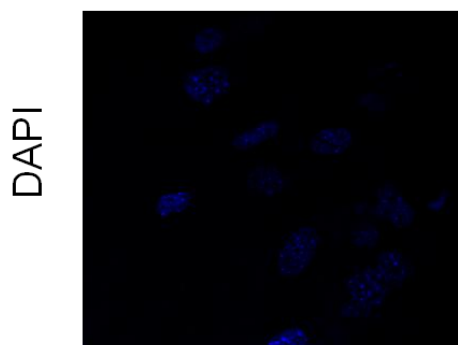
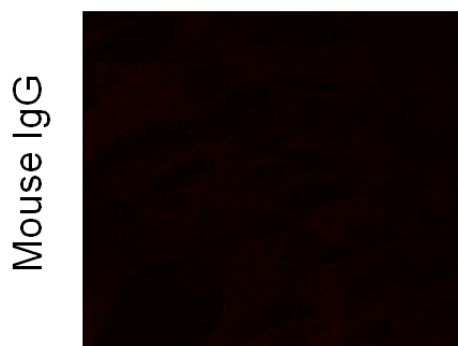
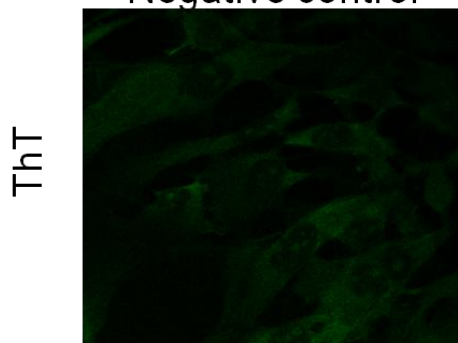
Figure 8. **Enhanced ThT fluorescence is detectible in mouse models of hepatic ER stress.** Liver sections were prepared from 15 week old mice supplemented with glucosamine (5% w/v) [25] and/or 4PBA (1g/kg body weight) [26] in the drinking water, starting at 5 weeks of age. Sequential sections were stained with 500 μ M ThT or anti-KDEL as indicated and fluorescent images were captured. * $P < 0.05$, $n = 3$, Scale bar = 100 μ m



Beriault, D. and Werstuck, G. BBA Molecular Cell Research 2013

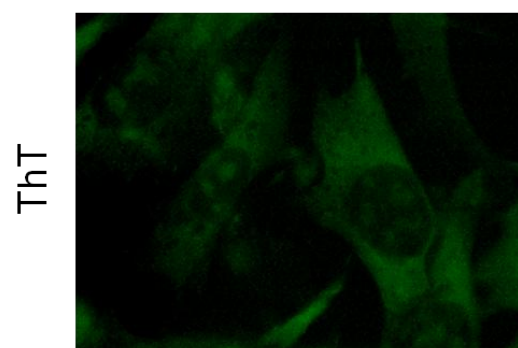
Supplemental Figure 2. **Additional controls for the ThT and KDEL staining in cell culture and mouse tissue.** A) 63x lens confocal images of MEFs stained with ThT and a mouse IgG (red) primary antibody (scale bar = 10 μ m). B) 100x lens epifluorescent images of MEFs stained with or without 5 μ M ThT. C) 10x lens epifluorescent images of apoE^{-/-} mouse liver stained with or without 500 μ M ThT (Scale bar = 100 μ m).

A) Mouse IgG (red)
Negative control



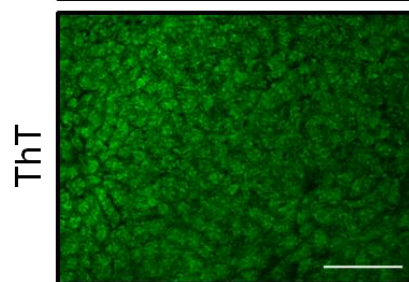
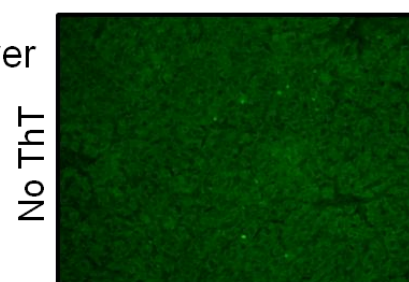
Confocal, 63x lens

B) Thioflavin T Negative control



Epifluorescent, 100x lens

C) ApoE^{-/-} Liver



Epifluorescent, 10x lens

4. Discussion

Thioflavin T (4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride) is a benzothiazole dye that exhibits enhanced fluorescence upon binding to proteins that are rich in β -sheet structures [11]. ThT has been shown to bind to amyloid fibrils containing “cross- β ” structures consisting of laminated β -sheets whose strands run perpendicular to the fibril. Such structures are associated with Alzheimer’s and prion diseases as well as systemic amyloidoses [23]. Upon binding to these structures, ThT exhibits a shift in emission maximum (from 445 nm to 482 nm) as well as an increase in fluorescence intensity of several orders of magnitude [12,13]. Together, these characteristics have made ThT a commonly used dye, both *in vitro* and *in vivo*, in the detection, diagnosis and analysis of amyloid fibrils and their associated pathologies in brain tissue and pancreatic islets [14,27,28].

Studies investigating the specificity of ThT have revealed that, in addition to amyloids, ThT can interact and bind to hydrophobic pockets in globular proteins. In particular, interactions with the α -helices of acetylcholinesterase [29], and the hydrophobic pocket of human serum albumin [30] are well documented. Furthermore, reports have shown that ThT can promote [31] or inhibit [32] protein aggregation suggesting that, under certain conditions, the interaction of ThT with protein structures can actively alter the folding process. Recently, ThT has been shown to interact with, and stabilize, DNA oligomers containing telemetric sequences in a pH and potassium-dependent manner [33]. The fluorogenic properties of ThT, together with its ability to

bind to non-amyloid structures suggests that we are only beginning to understand the utility of this small molecule.

In a typical cell, one third of all of the proteins that are synthesized, are processed in the ER. ER stress is a condition in which the capacity of the ER resident folding machinery is overwhelmed by the load of nascent proteins. This results in the accumulation of misfolded proteins and the subsequent activation of the UPR [2,3]. The UPR is initiated by three different trans-ER membrane proteins, PKR-like ER Kinase (PERK), Inositol-Requiring Enzyme (IRE)-1 and Activating Transcription Factor (ATF)-6, which act as sensors of misfolded proteins. Upon activation these factors act in concert to; i) reduce the load of nascent proteins that require folding by inhibiting protein translation, ii) increase the protein processing capacity of the ER by specifically enhancing expression of chaperones and foldases, and iii) increasing the capacity of the cells to degrade terminally misfolded proteins [2,3].

Generally ER stress is detected indirectly by measuring the levels of specific UPR factors. Commonly measured indicators of UPR activation include; phosphorylated PERK, phosphorylated eIF2 α , GADD153/CHOP, ATF4, Grp78/BiP, Grp94, calreticulin, and protein disulphide isomerase (PDI). These markers are usually detected by immunoblot or immunohistochemistry [6,34]. ER stress-induced XBP-1 splicing and UPR gene expression can also be quantified using RT-PCR. Although these detection techniques have been successfully employed in many studies, they do have several limitations; i) mRNA isolation/RT-PCR and immunoblot analysis can be time consuming and analysis is retrospective, ii) the sample being analyzed is generally destroyed by the

process of analysis, iii) other metabolic pathways can activate specific components of the UPR in the absence of ER stress thereby giving a false positive, iv) different cells/tissues express different sets of UPR genes and the detection methods have to be modified accordingly, and finally, v) all of these procedures are indirect measures of ER stress.

Our results suggest that ThT fluorescence is a viable new method to detect and quantify ER stress. The measured increase in ThT-fluorescence directly correlates to the degree of UPR activation, as quantified using more traditional assay systems. ThT has several apparent advantages over traditional methods including; i) enhanced ThT fluorescence is detectable 20 minutes after application of an ER stress-inducing agent, ii) ThT fluorescence can be continuously monitored and quantified in living cells over time, iii) ThT fluorescence a more direct measure of protein aggregation, and iv) ThT detection is effective in different cell types, that are challenged with a range of different ER stress-inducing agents and conditions.

At the present time, we do not know if the enhanced ThT fluorescence that is observed under conditions of ER stress is attributable to specific misfolded proteins or protein structures, or if the fluorescence is a result of non-specific binding to protein aggregates. Although we do present data showing that enhanced ThT fluorescence co-localizes with ER resident KDEL containing proteins under conditions of ER stress, we do not directly show that ThT is interacting with misfolded proteins in the ER. Previous reports have characterized the ability of ThT to bind to protein aggregates [11-14,32]. It should also be noted that ThT is not specifically localized to the ER and the accumulation of misfolded proteins in the cytosol, or in other subcellular organelles, may induce

enhanced ThT fluorescence. Furthermore, conditions of unconventional UPR that selectively activate only one ER stress sensor (PERK, IRE, or ATF6) independently of protein misfolding, will likely not induce enhanced ThT fluorescence.

5. Conclusions

Our results indicate that ThT fluorescence can be used to directly detect, monitor and quantify ER stress levels in living cells and in animal tissues. This technique has several advantages over the current established methods used to measure ER stress levels; it is inexpensive, allows for rapid analysis in living cells and in real time. Evidence supporting a role for ER stress in the development of human conditions and diseases including Alzheimer's, Parkinson's, diabetes mellitus, obesity, cancer and cardiovascular disease has increased interest in the detection of misfolded proteins in the ER. One potential application of ThT may be the detection and quantification of ER stress in tissues of cancer patients. A recent clinic study has found that the expression of Grp78 and CHOP in visceral adipocytes predicts endometrial cancer progression and patient survival [35]. Future studies will target this and other potential applications of ThT as a novel ER stress-sensing tool.

6. Competing interests

The authors have no competing interests to declare.

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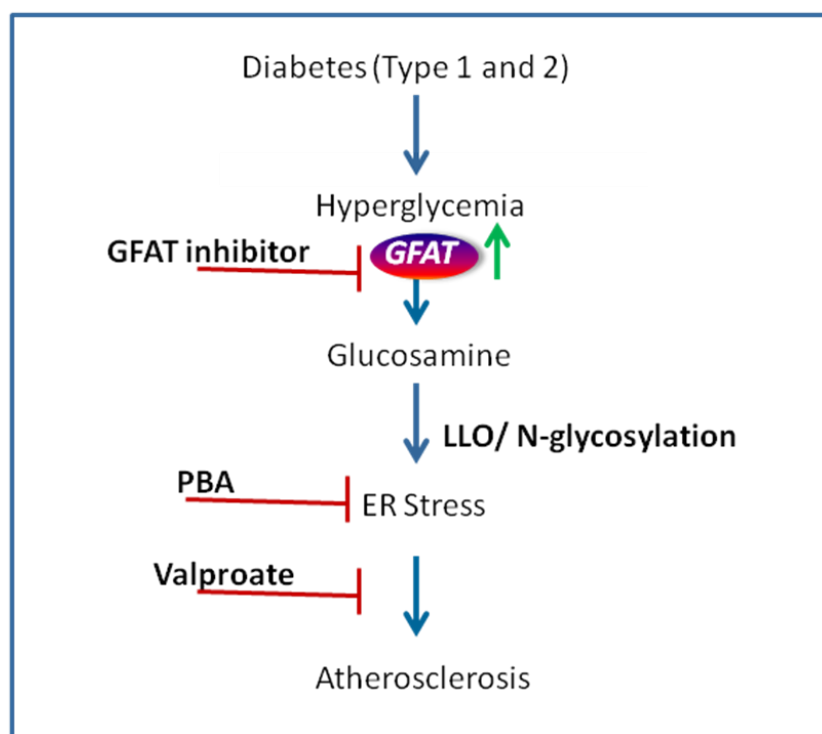
6 DISCUSSION

The hexosamine biosynthesis pathway (HBP) is an important branch of the glycolytic pathway. The HBP is well known for its ability to post-translationally modify proteins with N-acetyl-glucosamine on the hydroxyl group of Thr/Ser residues (known as O-linked glycosylation), and to co-translationally modify proteins on the amide nitrogen of Asn residues (known as N-linked glycosylation). These are distinct mechanisms that occur in the cytoplasm or endoplasmic reticulum, respectively. The HBP is thought of as a nutrient sensor as it links cellular metabolism directly to the proteome, which facilitates that metabolic information to the nucleus. O-glycosylation plays an important role in transcriptional regulation as it is found on many transcription factors and coactivators, and may even compete with phosphorylation for binding sites [108]. N-glycosylation plays an important role in protein folding, stability and secretion, as well as a role in cellular stress responses [109]. Disruptions in glucose metabolisms arising from disease states, including diabetes mellitus, can disrupt the HBP and potentially contribute to the associated pathophysiologies. The aims of this thesis were to determine whether increased flux through the HBP could promote ER stress and accelerated atherogenesis, and to investigate the physiological relevance of this effect in a hyperglycemic model of diabetes. We found that increased flux through the HBP disrupted lipid-linked oligosaccharide biosynthesis and protein N-glycosylation, which in-turn initiated ER stress and accelerated atherosclerosis (Figure 1). The levels of intracellular glucosamine, LLO intermediates, ER stress and atherosclerotic plaques were comparable between glucosamine-supplemented and hyperglycemic mice. Unlike the hyperglycemic mice,

glucosamine-supplemented and control mice had similar glucose and insulin levels, as well as a similar tolerance to glucose measured by an oral glucose tolerance test. Our studies suggest that supplementing mice with glucosamine allows us to mimic the condition of enhanced HBP flux observed in diabetic/hyperglycemic mice, without the added pathophysiological effects of hypoinsulinemia and dysglycemia. Therefore, this glucosamine-supplementation model may be useful in testing the role of the HBP in other complications associated with diabetes.

Figure 1. **Summary our working model of hyperglycemia-induced accelerated atherosclerosis.** High blood glucose increases the intracellular concentration of glucosamine, which can disrupt lipid-linked oligosaccharide biosynthesis and N-glycosylation, and cause ER stress and accelerate atherosclerosis. Potential agents that regulate this mechanism (ie. GFAT inhibitors, PBA, Valproate) may be useful therapeutics to slow the development of atherosclerosis in diabetic patients.

Summary of working model



There are still important questions regarding the relevance of our glucosamine model in a physiological setting. It has been argued that 1-5 mM glucosamine in cell culture models and mice supplemented with 5% w/v glucosamine may potentially be a super-physiological condition. Our reviewers tend to ask how the blood concentration of glucosamine compares between glucosamine-supplemented and hyperglycemic mice. The correct answer is that they do not compare. The conversion of glucose to glucosamine occurs as an intracellular function through the hexosamine biosynthesis pathway. Therefore, it would be better to compare the levels of intracellular glucosamine between our glucosamine-supplemented and hyperglycemic mice. The current established method is to measure protein bound O-linked N-acetylglucosamine (O-GlcNAc) by immunoblot. This method is an indirect measure of intracellular glucosamine levels, however, it has been shown to correlate well [51]. Using this method, we found that our glucosamine-supplemented and hyperglycemic mice had similar levels of intracellular glucosamine in aortic and hepatic tissue (Chapter 1, Figure 1). Current advancements in intracellular glucosamine detection involve the measurement of UDP-GlcNAc levels by mass spectrometry (LC-MS or MALDI imaging-MS). Our lab is currently trouble shooting these higher resolution methods, and we believe they will be important for correlating levels of glucose to glucosamine in hyperglycemic mice, and accurately comparing intracellular glucosamine accumulation to ER stress activation and atherogenesis *in vivo*.

In our study we found that 4-phenylbutyric acid (4-PBA) it was able to mitigate the ER stress response caused by glucosamine and tunicamycin, however, it had no

apparent effect on LLO biosynthesis *in vitro* (Chapter 2, Figure 5A and B). These results are consistent with the currently accepted hypothesis that 4-PBA acts as a chemical chaperone to reduce ER stress. In other studies, 4-PBA has been shown to inhibit atherogenesis caused by ER stress [102] and other ER stress-induced pathophysiologies [99, 110]. We have also shown, in this thesis and other studies, that valproate can inhibit pro-atherogenic pathways caused by glucosamine- and hyperglycemia-induced ER stress and slow atherogenesis [93, 111]. Together, these results suggest that the downstream pathways activated by chronic ER stress play the major role in atherogenesis, rather than the effects on specific proteins. As several independent risk factors for cardiovascular disease, including dyslipidemia [72, 112], diabetes [43], hyperhomocysteinemia [68, 113], and hypertension [114] have all been associated with ER stress, our lab hypothesizes that ER stress may be a unifying and underlining mechanism of atherogenesis.

The potential role of glucosamine-induced ER stress in diabetic atherogenesis emphasizes the importance of the rate limiting enzyme, GFAT, which converts glucose to glucosamine. In cell culture, GFAT activity has been shown to play a central role in the ER stress pathway as GFAT inhibition can attenuate glucose-induced ER stress [43], and GFAT overexpression is sufficient to promote ER stress under normo-glycemic conditions [66]. The commonly used GFAT inhibitors in cell culture (ie. azaserine or 6-diazo-5-oxonorleucine) have very low specificity and high cytotoxicity [106]; and therefore are not ideal for use in mouse models. A specific GFAT inhibitor has been developed based on isoquinoline (Bolin *et al.*; patent No. US 7,037,924 B2). Dr. Lisa

Walter, a previous graduate student under the joint supervision of Dr. Capretta and Dr. Werstuck, has developed derivatives of this compound that effectively inhibit recombinant human GFAT *in vitro*. In collaboration with Dr. Walter, we were able to further evaluate these derivatives in cell culture to determine the lethal dose 50 (LD50) and inhibition kinetics. This project will begin to test the therapeutic potential of targeting GFAT to reduce ER stress and atherogenesis caused by hyperglycemia.

The central role of macrophage/foam cells in atherosclerotic development has been established over the last decade. Not only do they play an important role in efferocytosis, the inflammatory response and the recruitment of immune cells [115, 116], but they have been shown to have high metabolic activity [117] and be susceptible to ER stress [118]. In fact, studies suggest that ER stress plays a major role in macrophage apoptosis and the progression of atherosclerosis [69, 102, 103]. A continuation of these studies could test the role of the hexosamine biosynthesis pathway in macrophage ER stress activation in hyperglycemic mice. This could involve mouse bone marrow transplantation experiments using either lentiviral vectors that overexpression or knockdown (shRNA) GFAT in myeloid cells of hyperglycemic and control mice. Such lentiviral vectors have been obtained by Dr. Geoff Werstuck's laboratory from Santa Cruz Biotech and are currently being tested.

It is interesting to note that the hyperglycemic and hypoglycemic conditions may have a similar effect on lipid-linked oligosaccharide biosynthesis. It has been shown that a decrease in flux through the HBP can also disrupt LLO biosynthesis [119-121]; and that hypoglycemia can cause ER stress [122-124]. In fact, loss of function mutations in GFAT

cause disruptions in LLO biosynthesis, which can be corrected for by supplementing with low concentrations of glucosamine [121]. Furthermore, Tunicamycin, a UDP-GlcNAc analog, inhibits the flux through the first enzyme (GPT) of the LLO pathway [125] and therefore may mimic the LLO pathway under a hypoglycemic condition. Insulin resistance is a condition associated with significantly reduced levels of glucose uptake into skeletal muscle cells and adipocytes: cells that rely upon insulin-dependent glucose uptake [126, 127]. It is possible that insulin resistance affects LLO biosynthesis in these cells. All together, these studies suggest that a variety of conditions of glucose dysregulation, including hyperglycemia, hypoglycemia, and/or insulin resistance, may share a similar mechanism to activating ER stress, by disrupting LLO biosynthesis.

The identification of a role for the HBP-LLO-ER stress pathway in diabetes associated accelerated atherosclerosis identifies new targets for potential therapeutic intervention. In fact, GFAT inhibitors are now being tested in our mouse models of hyperglycemia and atherosclerosis. However, the central importance of the HBP in essential cellular processes means that great care will have to be taken in the manipulation of this pathway and it is very possible that GFAT inhibitors will never be clinically useful. Validation of this pathway may however lead to the identification of other factors within this process that can be more subtly manipulated. Alternatively, recognition of a role for HBP-LLO-ER stress may be important in the development of novel biomarkers of disease development and progression.

Advancements in ER stress detection will be important to any future clinical application. Currently, the classical methods of detection involve measuring mRNA or

protein levels of markers of the unfolded protein response. However, there are limitations to these methods. One example of this is that measuring markers of the unfolded protein response is an indirect measure of unfolded proteins and ER stress. Therefore, absolute levels of misfolded proteins/ER stress cannot be quantified. Another issue is that the expression of these markers can vary between cell types and the expression pattern can change depending on the type of ER stress: acute vs. chronic [128]. In this thesis we have identified a fluorescent probe (Thioflavin T) that can detect misfolded/aggregated proteins in the ER during ER stress. Although the assay also has its own limitations (discussed in chapter 3), it may provide a building block for future ideas or even the development of efficient derivatives. One example of this is that Thioflavin T is a positively charged molecule at pH 7. Negatively charged amino acids like aspartic acid and glutamic acid are polar residues that tend to be present on the outer surface of properly folded proteins. Therefore, when staining for unfolded proteins with Thioflavin T these charged amino acids could theoretically increase background 'noise' in the fluorescent stain due to ionic interactions. It is possible that non-ionic derivatives of Thioflavin T may increase the specificity for the hydrophobic regions of misfolded/aggregated proteins. Commercial non-ionic derivatives of Thioflavin T do exist, however, they are quite expensive. Another idea is to add a KDEL-tag to Thioflavin T. KDEL (Lys-Asp-Glu-Leu) is a retention sequence for the endoplasmic reticulum, and therefore, may keep the majority of Thioflavin T molecules localized to the ER. At the moment Thioflavin T is not specific to ER stress as it will also bind

misfolded proteins in the cytoplasm. However, the addition of a KDEL-tag may allow for Thioflavin T to be used as a ER stress-specific indicator.

In conclusion, the results of this thesis suggest that the hexosamine biosynthesis pathway may play a critical role in the development of diabetic atherosclerosis. There are many interesting questions that remain before such a hypothesis could be tested at a clinical level. Further studies investigating intracellular glucosamine levels in diabetic tissue by mass spectrometry, small molecule GFAT inhibitors, and the relevance of the hexosamine pathway in macrophage apoptosis and atherosclerosis would be an important step towards such a goal. The World Health Organization (WHO) estimates that the number of people with diabetes will double between 2005 and 2030. Therefore, understanding the mechanisms of diabetic complications could play a major role in reducing the social and economic burden this disease will have on health care systems around the world over the next few decades.

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