GFAT AND GLYCOSYLATION IN HYPERGLYCEMIA-ASSOCIATED ER STRESS

THE ROLE OF GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE AND PROTEIN GLYCOSYLATION IN HYPERGLYCEMIA-ASSOCIATED ENDOPLASMIC RETICULUM STRESS

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Abstract

Diabetes mellitus is a major independent risk factor for cardiovascular disease (CVD) and stroke, however the cellular mechanisms by which diabetes contributes to vascular dysfunction are not fully understood. In recent decades, multiple molecular mechanisms have been implicated in hyperglycemia-associated vascular damage and CVD [1]. It is well established that hyperglycemia promotes intracellular glucose flux through the hexosamine pathway where the rate-limiting enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT) produces glucosamine-6-phosphate [2,3]. We have shown that elevated levels of intracellular glucosamine cause ER stress and activation of the UPR in multiple cell types [4]. Additionally, we have previously shown that ER stress is associated with lipid accumulation, activation of inflammatory pathways, and is associated with atherosclerotic plaque formation in hyperglycemic mice [4,5]. We hypothesize that the accumulation of intracellular glucosamine, observed in conditions of hyperglycemia, promotes atherogenesis via a mechanism that involves the hexosamine pathway, protein glycosylation and ER stress.

Using *in vitro* over-expression studies, we investigated the role of GFAT in hyperglycemia-associated ER stress. We developed methods to increase GFAT expression in both HepG2 cells and HASMC. However, we found that GFAT over-expression is insufficient to induce an ER stress response. Further investigation of this system suggests that the over-expressed GFAT does not increase intracellular glucosamine levels to sufficiently promote ER stress.

We have also investigated the role of protein glycosylation in glucosamine-induced ER stress. We have shown that *O*-linked glycosylation plays a role in ER stress induction. We have also shown that *N*-linked protein glycosylation is affected by elevated cellular glucosamine levels. Thus, dysregulated glycosylation of newly synthesized proteins may contribute to the accumulation of unfolded protein in the ER and lead to the activation of the UPR.

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Foremost, I would like to offer my sincerest gratitude to my supervisor, Dr. Geoff Werstuck. His encouragement and guidance have been invaluable throughout my graduate experience. His ability to offer direction and guide learning, while still being a receptive and open teacher has enabled me to become a more confident and knowledgeable scientist. His enthusiasm has continually piqued my own interest in science, and made me passionate about contributing to research.

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List of Abbreviations

ATF6	Activating Transcription Factor 6
ATP	Adenosine Triphosphate
AGEs	Advanced Glycation End-products
CVD	Cardiovascular Disease
CDG	Congenital Disorders of Glycosylation
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
eIF2alpha	Eukaryotic Initiation Factor 2 alpha
GLUT	Glucose Transporter
Grp78/BiP	Glucose-regulated Protein 78
GFAT	Glutamine:Fructose-6-phosphate Amidotransferase
HDL	High Density Lipoproteins
HRP	Horseradish Peroxidase
HASMC	Human Aortic Smooth Muscle Cells
HEK 293	Human Embryonic Kidney 293
HepG2	Human Hepatocarcinoma Cells
hSR-BI	human Scavenger Receptor – BI
IRE1	Inositol Requiring Endoribonuclease 1
IL-1	Interleukin-1
ICAM	Intracellular Adhesion Molecule
LOCAT	Lopid Coronary Angiopathy Trial
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein receptor
MOBIXLab	Molecular Biology and Biotechnology Laboratory
mSR-BI	mouse Scavenger Receptor – BI
O-GlcNAcase	*N*-acetyl-β-D-glucosaminidase
NAD^+ and $NADP^+$	Nicotinamide Adenine Dinucleotide
OGT	*O*-GlcNAc Transferase
O-GlcNac	*O*-linked *N*-acetylglucosamine
PBS	Phosphate Buffered Saline
PERK	Protein Kinase R-like ER Kinase
ROS	Reactive Oxygen Species
SMC	Smooth Muscle Cells
SREBPs	Sterol Element Regulatory Binding Proteins
TIC	Trypsin Inhibitory Capacity
TNFα	Tumour Necrosis Factor α

List of Abbreviations (continued)

T1D	Type I diabetes
T2D	Type II diabetes
UPR	Unfolded Protein Response
UDP	Uracil-diphosphate
VCAM	Vascular Cell Adhesion Molecule
VLDL	Very Low Density Lipoproteins
XBP1	X-box Binding Protein 1
α_1-AT	α_1-antitypsin

1.0 Introduction

1.1 Preface

The word *metabolism* originated from the Greek word *metabole* meaning, "to change from". It is a simple definition, but it is this ability "to change" that has enabled the evolution of life as we know it. Few cellular processes are as elegantly choreographed, as our ability to break down, and synthesize macromolecules such as sugar, fat and protein – a dynamic process, providing both the energy and chemical building blocks required for survival. We are so dependent on the process of cellular metabolism, that it is not surprising that its dysfunction has become the cause of some of our most severe disease states. Diabetes mellitus is one of the most commonly known conditions resulting from a heterogeneous group of metabolic disorders. It is associated with multiple, severe complications – including blindness, renal failure and neuropathy. Diabetes is also an independent risk factor for cardiovascular disease (CVD) [1]. Diabetics have a 2-4 fold increased risk of developing CVD and it is estimated that 50-80% of diabetics will die as a result of myocardial infarction or stroke – making it the leading cause of death among individuals with diabetes [6,7]. The established correlation between the incidence of CVD and diabetes has propelled the research community to investigate the molecular and cellular mechanisms by which diabetes mellitus promotes atherosclerosis. These investigations will enable further understanding of these two conditions, as well as potentially identify novel methods to treat and prevent diabetes-associated CVD. The impact of these studies is far reaching, as they will aid in the

management of these diseases and ultimately affect world health and mortality in the decades to come.

1.2 Diabetes Mellitus

Recent decades have seen a significant worldwide increase in the incidence of diabetes mellitus. It currently affects 150 million people – though this number is projected to reach 300 million by the year 2025 [6]. Augmented by the growing prevalence of obesity, sedentary lifestyle and poor eating habits, diabetes is quickly becoming a worldwide epidemic.

The pathologic definition of diabetes mellitus describes it as a metabolic disorder in which carbohydrate, fat and protein metabolism is disrupted due to defects in insulin secretion and utilization [8]. It has multiple etiologies, giving rise to three distinct forms of the condition. Type I diabetes (T1D) accounts for roughly 10% of cases[6,7]. It is an autoimmune disease resulting in β-cell death in the pancreas, and decreased insulin secretion. Daily administration of exogenous insulin is required for these individuals to survive. Type II diabetes (T2D) accounts for the remaining 90% of cases[6,7]. It is characterized by a combined resistance of peripheral tissues to insulin and impaired β-cell function in the pancreas. This form has a slower onset and is commonly associated with obesity. T2D diabetes commonly affects adults, however the incidence in children continues to increase as the rate of childhood obesity continues to climb. The third form of diabetes, gestational diabetes, is a short-term condition associated with roughly 3.5% of all pregnancies[7]. Women who experience gestational diabetes have an increased risk

of developing T1D or T2D with age. Gestational diabetes can also result in a number of complications for the infant after birth, including breathing problems, obesity and glucose intolerance later in life[6].

Despite the distinct differences between the three forms of diabetes, they are all characterized by chronic hyperglycemia. Hyperglycemia is now widely accepted as the causative factor in diabetes-associated complications. Despite advances in insulin supplementation and attempts to tightly control plasma glucose levels, complications continue to plague individuals with diabetes, suggesting that even short-term deviations in the control of blood glucose can have significant effects. Diabetic complications include retinopathy, renal failure, peripheral nerve dysfunction and CVD, all of which are manifestations of vascular disease. These observations have led to studies investigating why hyperglycemia has such a significant impact on vascular function.

1.3 Atherosclerosis

Atherosclerosis is a disease of the large, muscular arteries which contributes greatly to the overall pathogenesis of cardiovascular disease. It is often defined as an inflammatory response to arterial injury and is characterized by lipid deposition and plaque formation in artery wall. Over time, this can lead to advanced lesion formation. Complications associated with lesion formation and rupture are the underlying causes of myocardial infarction and stroke.

Plaque formation is initiated by insult to the vascular endothelium, resulting in activation of inflammatory pathways. This results in the increased expression of cell

adhesion molecules, vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM), E-selectin and P-selectin [9]. Monocytes do not normally adhere to the endothelium, but the expression of adhesion molecules enables them to bind and migrate into the intimal space of the artery, through a process known as "rolling" [10]. These monocytes differentiate into macrophages which take up and accumulate lipid from oxidized low density lipoprotein (LDL) particles. The uptake of oxidized LDL by macrophages is mediated by scavenger receptors, including CD36 [10,11]. The lipid engorged macrophages are the most prominent cells in early lesions or fatty streaks. The lipid droplets in their cytoplasm appear foamy under the electron microscope and they are often referred to as foam cells [11]. Further lesion development and growth is characterized by foam cell apoptosis and cytokine secretion. In particular, oxidized LDL activates foams cells to produce the pro-inflammatory cytokines, tumour necrosis factor α (TNFα) and interleukin-1 (IL-1) [10,12]. These stimulate the migration of smooth muscle cells (SMC) from the medial layer of the artery into the intima. SMC secrete collagen, eventually giving rise to a fibrous-caped lesion with a pro-thrombogenic necrotic core. Lesion rupture results in platelet adherence and clot formation – potentially leading to vessel occlusion – and is the pathogenic cause of heart attack and stroke [10,13,14,15].

The established correlation between diabetes mellitus and CVD has led to a number of hypotheses as to how hyperglycemia may induce the microvascular and macrovascular damage. These mechanisms are derived from the effects of elevated levels of glucose and include increased polyol pathway flux, the effects of advanced

glycation end-products (AGEs), activation of protein kinase C and increased hexosamine pathway flux [1,16]. The experimental research studying these mechanisms, using cell culture systems and animal models, has to date suggested that hyperglycemia induces over-production of superoxide by the mitochondrial electron transport chain [1]. Consequently, oxidative stress is theorized to be a potential mechanism by which hyperglycemia is implicated in the various diabetic complications and vascular dysfunction. Multiple clinical studies have been performed to determine whether anti-oxidant treatment can alleviate the deleterious effects of reactive oxygen species (ROS) and protect against the development of CVD. From 1997 through to 2005, multiple studies investigated the effects of vitamin E, vitamin C and β-carotene supplementation on cardiovascular risk [17,18,19,20,21,22]. These studies all found that the anti-oxidants had no effect on reducing CVD [17,18,19,20,21,22]. The paradox which developed from the ineffectiveness of antioxidant treatment suggested that other cellular mechanisms may be acting in concert with oxidative stress to promote atherosclerosis. One hypothesis is that increased glucosamine production, resulting from hyperglycemic conditions, contributes to the progression of atherosclerosis.

1.4 Glucose, Glucosamine, the Hexosamine Pathway

The metabolism of glucose is essential to survival. It provides a source of energy, in the form of ATP as well as cofactors, such as nicotinamide adenine dinucleotide (NAD^+ and $NADP^+$) [23]. These macromolecules are required in a plethora of cellular processes – from protein signaling cascades, to the synthesis of DNA and fatty acids.

The metabolism of glucose also provides many of the chemical building blocks required for the synthesis of a number of cellular components, including the synthesis of amino acids and the processing and storage of fat. We are so reliant on glucose, as an energy source, that the cell will break down non-essential protein and fat, in periods of glucose starvation, in order to make glucose via gluconeogenesis [23]. Consequently, it is not surprising that glucose metabolism is a highly evolved process. It becomes even more complex when studying organisms, where multiple organs, tissues, and cell types play a role in the process.

The metabolism of glucose begins with the breakdown of carbohydrate into simple sugar, absorption of sugar into the bloodstream, and its delivery to target tissues – all of which are mediated by the dynamic interplay of hormones, insulin and glucagon, which act to maintain a steady blood glucose level. While tissues and cell types vary in the body, the metabolism of glucose at the cellular level, begins with the same event – its import into the cell via a glucose transporter (GLUT) protein (Figure 1.0). While many GLUT proteins have been identified to date, the most significant of these are the high affinity GLUT 1 and GLUT 4 transporters as well as the low affinity GLUT 2 [24]. GLUT 1 is the primary glucose transporter and provides cells with basal glucose requirements [25]. GLUT 4 is responsible for insulin-mediated glucose uptake in skeletal muscle, heart and adipose tissue [24,26,27]. GLUT 2 is present on pancreatic β cells and tissues exposed to large glucose fluxes, such as liver and kidney [28]. GLUT 2 has a higher Km for glucose than GLUT 1 [29,30]. Consequently, the rate of glucose transport by GLUT2 is more sensitive to the ambient glucose concentration than GLUT1 – a

characteristic that enables cells utilizing GLUT2 to be highly sensitive to hyper- and hypo- glycemic conditions [29,30].

Immediately following transport into the cell, hexokinase converts glucose to glucose-6-phosphate to prevent its reverse translocation across the plasma membrane [24]. At this stage, glucose has many possible cellular fates. It can be metabolized through glycolysis and the citric acid cycle to provide the cell with ATP energy requirements and a number of required metabolites [24]. It can also be converted to glycogen for storage and later use [24]. In some tissues, glucose-6-phosphate is shunted through the pentose phosphate pathway to supply the cell with NADPH [24]. Glucose can also be converted into a number of UDP-conjugated sugars and used for protein glycosylation [31,32]. Lastly, glucose-6-phosphate can be converted to fructose-6-phosphate and shunted through the hexosamine pathway (Figure 1.0) [24]. In the hexosamine pathway, fructose-6-phosphate is used as substrate for the production of UDP-*N*-acetylglucosamine, a metabolite required for *O*-linked and *N*-linked protein glycosylation [24]. The enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) is the rate limiting enzyme involved in this process [3]. GFAT is a 77 kDa cytosolic protein that has both glutaminase and isomerase functions [3,33,34]. It facilitates the conversion of fructose-6-phosphate to glucosamine-6-phosphate [35]. The glutaminase function of the enzyme uses glutamine as an amine donor, producing glutamate [36]. The isomerase function of the enzyme converts pentose to hexose and the transfer of the amine group to the hexose produces glucosamine-6-phosphate [34,36].

There is much evidence in both cell culture and animal model systems implicating the hexosamine pathway in the pathogenesis of diabetes [37]. It is also now widely accepted that flux through the hexosamine pathway serves as a cellular glucose sensor, indirectly controlling glucose uptake and insulin sensitivity [37,2]. Under normal physiological conditions, about 3% of glucose is converted to glucosamine by GFAT [38]. However, under the hyperglycemic conditions seen in diabetes, the amount of glucose shunted through this pathway is increased and elevates cellular glucosamine levels [36,2]. Through a mechanism which remains unclear, high levels of glucosamine contribute to cellular dysfunction by interfering with protein folding in the endoplasmic reticulum (ER) [39]. The cellular effects of glucosamine and ER stress have been a neglected area of research in the context of diabetic complications. Consequently, it is hypothesized that the hexosamine pathway and GFAT may play a role in stimulating ER stress under hyperglycemic conditions [2,39]. The cellular implications of hexosamine pathway flux and ER stress make them highly probable contributors to the processes by which diabetes promotes CVD.

Figure 1.0 The Hexosamine Pathway and Hyperglycemia-induced ER Stress
Hyperglycemic conditions cause glucose to be shunted into the hexosamine pathway, increasing cellular levels of glucosamine. Through an unknown mechanism, glucosamine induces a state of ER stress, which contributes to lipid accumulation, inflammation and apoptosis - leading to atherosclerosis.

1.5 Endoplasmic Reticulum Stress

The ER is the site of mRNA translation, as well as folding and modification of proteins destined for secretion or association with the cell surface. An accumulation of unfolded proteins in the lumen of the ER due to compromised protein folding or protein degradation pathways results in a state of ER Stress [39,40,41]. It is brought on by a number of physiological conditions, one of which is increased cellular glucosamine levels[39]. The cellular response to ER stress is known as the unfolded protein response (UPR) (Figure 2.0) [39,40,41]. The UPR is primarily facilitated by the ER-lumenal protein GRP78. It is theorized that GRP78 associates with the ER membrane-bound proteins PERK, ATF6 and IRE1 under normal conditions of ER homeostasis [41,42,43]. However, GRP78 dissociates from PERK, ATF6 and IRE1 when mis-folded proteins accumulate, thus enabling each protein to facilitate its specific role in alleviating ER stress [41]. GRP78 is a protein chaperone which associates with unfolded proteins, preventing them from aggregating [39,41]. This is essential to facilitate protein folding, as well as enable degradation of terminally mis-folded proteins since heavily aggregated protein cannot be degraded by the ER-associated degradation (ERAD) pathway [44]. Activated PERK inhibits eIF2α, resulting in attenuation of protein translation [45]. ATF6 translocates to the Golgi and site-directed cleavage releases the N-terminal transcription activation domain that specifically increases expression of genes encoding ER chaperones [43,46,47]. Lastly, activation of IRE1 enables the RNase activity of the enzyme [48]. IRE1 facilitates the cleavage of XBP1 mRNA [49,50,51] . The spliced

mRNA is translated into an active transcription factor involved in upregulating both GRP78 expression as well as proteins involved in ERAD[49,50,51].

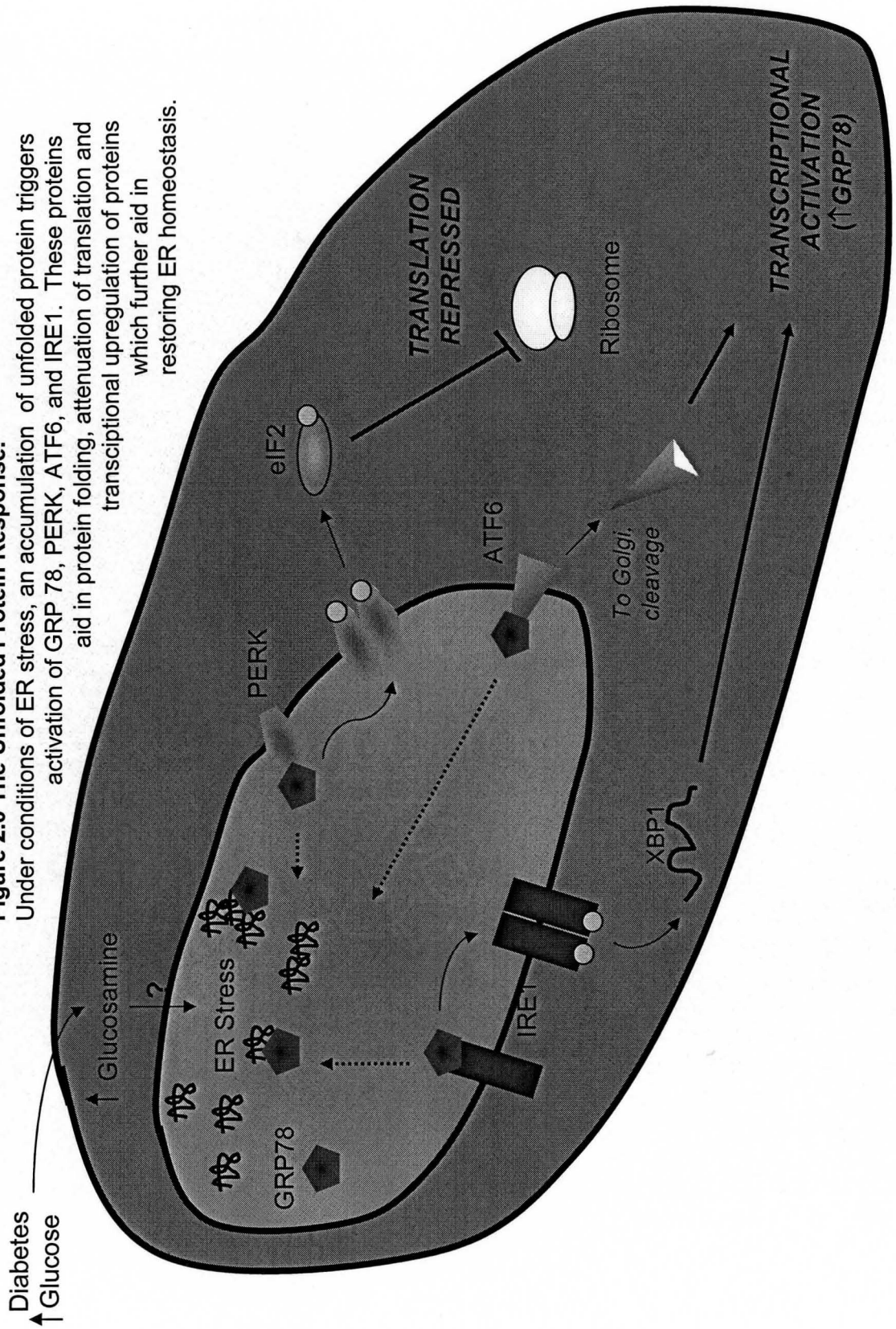


Figure 2.0 The Unfolded Protein Response.
Under conditions of ER stress, an accumulation of unfolded protein triggers activation of GRP 78, PERK, ATF6, and IRE1. These proteins aid in protein folding, attenuation of translation and transcriptional upregulation of proteins which further aid in restoring ER homeostasis.

Interestingly, ER stress has also been implicated in both obesity and cellular events associated with atherosclerosis – both complications associated with diabetes mellitus. ER stress has also been implicated in the induction of insulin resistance in obese mice [52]. Obesity-induced ER stress inactivates cell signaling downstream of the insulin-receptor, resulting in a down-regulation of both glycolysis, glycogen synthesis and recruitment of GLUT 4 to the plasma membrane [52,53]. The increased occurrence of diabetes in obese individuals and the involvement of ER stress in insulin-resistance further supports the theory that ER stress is involved in the pathogenesis of diabetes mellitus. Severe ER stress has also been shown to lead to a variety of cellular events associated with atherosclerosis. Most severely, ER stress can induce apoptosis, through activation of the caspase signaling cascade [54,55]. ER stress has also been shown to activate sterol regulatory element binding proteins (SREBPs), which activate fatty acid and cholesterol biosynthesis as well as expression of the LDL receptor [54,56]. This is of significance due to the role of lipid accumulation and foam cell formation in atherosclerotic plaque development. Based on these studies, ER stress is now widely accepted as a cellular event associated with both diabetes and atherosclerosis.

1.6 Protein Glycosylation

It is known that high levels of glucosamine contribute to cellular dysfunction by interfering with protein folding and induce a state of ER stress [39]. In particular, it has been shown that glucosamine can induce ER stress in cell types relevant to the development of atherosclerosis [4]. However, the mechanism by which glucosamine

exhibits this effect remains undefined. Glucosamine plays a fundamental role in both *N*-linked and *O*-linked protein glycosylation. Consequently, it has been hypothesized that glucosamine may induce ER stress through a mechanism involving protein glycosylation.

O-linked glycosylation refers to the addition of *O*-linked *N*-acetylglucosamine (*O*-GlcNac) to proteins on serine and threonine residues (Figure 3.0) [57]. While the role of this type of glycosylation is still largely unclear, there is a growing body of evidence suggesting that *O*-GlcNAc is involved in cellular signaling [53,57,58]. It has been suggested that its function may even be analogous to phosphorylation with respect to its ability to regulate enzyme activity and enable protein-protein interactions [57]. The addition of *O*-GlcNAc to proteins is facilitated by the enzyme *O*-GlcNAc transferase (OGT) [57]. UDP-GlcNAc is the required substrate of the enzyme and it is added to many nuclear and cytosolic proteins. As mentioned, *O*-linked glycosylation is much like phosphorylation. The addition of *O*-GlcNAc is a dynamic and regulated process, and proteins can also be deglycosylated. This is facilitated by the enzyme *N*-acetyl-β-D-glucosaminidase (*O*-GlcNAcase) [57].

Multiple inhibitors of both OGT and *O*-GlcNAcase exist. The compound, alloxan is a potent inhibitor of OGT and has been shown to cause beta-cell death in the pancreas [59]. It is commonly used in animal models, as a method of inducing type I diabetes [59]. In addition to inhibiting OGT, however alloxan is also an inhibitor of glucokinase [59].

There are also inhibitors of *O*-GlcNAcase. PUGNAc and "9c" (see Materials and Methods) are potent inhibitors of *O*-GlcNAcase. PUGNAc has been shown to also

inhibit β-hexosaminidase, whereas "9c" is a more selective inhibitor for *O*-GlcNAcase, than β-hexosaminidase [60]. All of these compounds have become useful in the study of *O*-linked glycosylation.

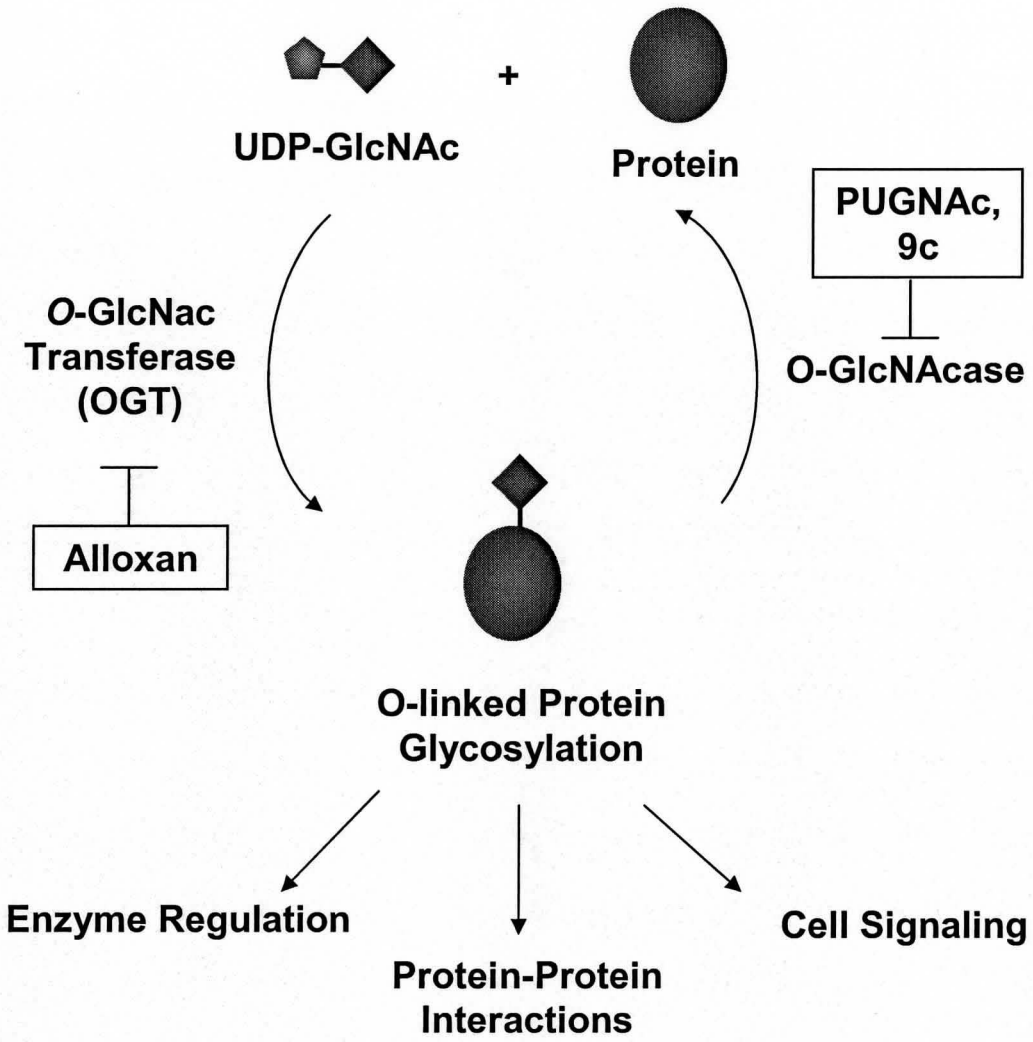


Figure 3.0 *O*-linked Protein Glycosylation.
O-linked glycosylation refers to the addition of *O*-linked *N*-acetylglucosamine (*O*-GlcNac) to proteins on Ser and Thr residues. UDP-GlcNAc is used to add *O*-GlcNAc to proteins and is facilitated by the enzyme *O*-GlcNAc Transferase (OGT). Proteins can also be deglycosylated and this is facilitated by the enzyme *N*-acetyl-β-D-glucosaminidase (*O*-GlcNAcase). *O*-linked glycosylation is involved in cellular signaling and its function may be analogous to protein phosphorylation with respect to its ability to regulate enzyme activity and enable protein-protein interactions.

Multiple inhibitors of both OGT and *O*-GlcNAcase exist. The compound, alloxan, is a potent inhibitors of OGT. PUGNAc and "9c" are potent inhibitors of *O*-GlcNAcase.

N-linked glycosylation refers to the co-translational addition of a branched sugar moiety to proteins synthesized in the ER (Figure 4.0). *N*-linked glycosylation has a number of roles intracellularly, but its primary involvement in the ER is to facilitate folding of newly synthesized proteins.

The process of *N*-linked glycosylation begins with the construction of a 14-saccharide unit (Glc_3-Man_9-$GlcNAc_2$) on a lipid carrier, by a series of glycosyltransferases in both the cytoplasm and ER lumen [32]. The saccharide unit is then transferred to proteins on the Asparagine of an Asn-X-Ser/Thr sequence as proteins are translated [61]. The *N*-linked glycan aids in protein folding by enabling association with ER chaperones, Calnexin and Calreticulin [61]. A cycle of deglucosylation and reglucosylation occurs until the protein is correctly folded [61]. If the protein cannot be folded correctly, glycation is trimmed to label the protein for ERAD [61]. These proteins are translocated out of the ER and degraded by the 26S proteasome [61].

Correctly folded proteins are translocated to the Golgi, where they are further processed. Three glucose units and up to six mannose residues can be removed from the glycan [32]. GlcNAc, galactose, sialic acid and fucose residues can then be added to create multiple, differentially branched glycan forms [61].

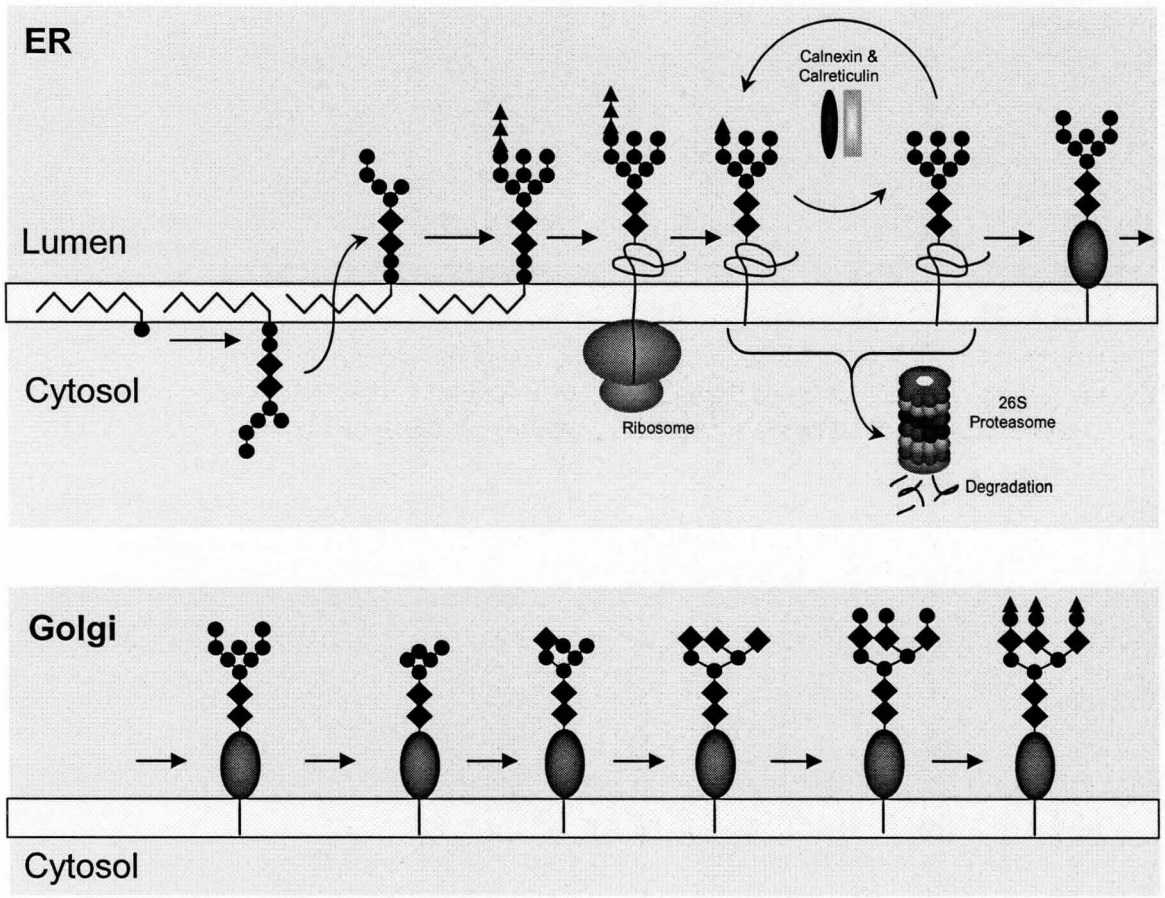


Figure 4.0 *N*-linked Glycosylation and Protein Folding.
The process of *N*-linked glycosylation begins with the construction of a 14-saccharide unit on a lipid carrier, by a series of glycosyltransferases in both the cytoplasm and ER lumen. The saccharide unit is then transferred to proteins as they are translated. The *N*-linked glycan aids in protein folding by enabling association with ER chaperones. A cycle of deglucosylation and reglucosylation occurs until the protein is correctly folded. If the protein cannot be folded correctly, it targeted for ER-associated degradation (ERAD). These proteins are translocated out of the ER and degraded by the 26S proteasome.

Correctly folded proteins are translocated to the Golgi, where they are further processed. Three glucose units and up to six mannose residues can be removed from the glycan. GlcNAc, galactose, sialic acid and fucose residues can then be added to create multiple, differentially branched glycan forms.

Like the genome and proteome, the study of the "glycome" has become an intense area of research. A single protein can have a wide array of glycans attached to it, yielding multiple "glycoforms" of the same protein [32]. As a result, the glycome is suggested to be as much as 10000 times larger than the proteome itself [32].

Given that *N*-linked protein glycosylation plays an important role in protein folding and synthesis, it is not surprising that a number of glycosylation disorders have been identified. The term congenital disorders of glycosylation (CDG) is used to describe this rapidly expanding collection of conditions which include defects in lipid-linked oligosaccharide synthesis as well as defects in the processing of protein-bound sugar chains [32]. There is a wide clinical variation between CDG types, depending on the nature of the defect and the mutant enzyme involved. Some forms of CDG cause premature death, where others are well tolerated [32]. Some tissues are affected more than others by the defects. Tissues with high glycosylation demands, rapid protein turnover or that are highly secretory are more sensitive to defective *N*-linked glycosylation [32]. In particular, hepatocytes, enterocytes and leukocytes are known to be largely affected [32]. The impact of these defects further highlights the importance of *N*-linked glycosylation to protein synthesis, processing, ER homeostasis and general cellular function.

1.7 Protein Glycosylation, ER Stress and Diabetes

Hyperglycemia increases the cellular level of glucosamine, and thus could have significant effects on both *O*-linked and *N*-linked protein glycosylation. This is true for

O-linked glycosylation. Hyperglycemia and increased levels of glucosamine have been shown to increase the cellular level of *O*-linked glycosylation [59]. Of particular note, it has been shown that hyperglycemic conditions result in increased glycosylation of the transcription factor, Sp1, inhibiting its activity and as well as its degradation by the 26S proteasome [53,57,58]. Glycosylation of the proteasome itself has also been shown to decrease its protein degradation capabilities [58]. Based on these observations, it is hypothesized that increased *O*-linked glycosylation of proteins may have an effect on protein degradation, resulting in their accumulation and the induction of ER stress and the UPR.

With respect to *N*-linked glycosylation, increased glucose flux through the hexosamine pathway could conceivably dysregulate polysaccharide production. This would have drastic effects on protein glycosylation and protein folding and would be a possible explanation for the induction of ER stress and the UPR under hyperglycemic conditions. It has been shown that glucosamine has the capacity to prevent the synthesis of a variety of glycoprotein-containing viruses in a variety of infected cells types [62]. Glucosamine prevents synthesis of influenza virus, Semliki Forest virus and other enveloped viruses, suggesting glucosamine has the ability to affect glycoprotein production [62].

With regards to glucosamine-induced ER stress, one or both types of protein glycosylation may play a role in the process. Investigating these types of glycosylation present a variety of challenges. In the case of *O*-linked glycosylation, it is difficult to investigate the glycosylation state of a protein without using complex mass spectrometry

methods. Additionally, a single *O*-GlcNAc has little effect on the molecular weight of the protein, making it difficult to use immunoblotting as a tool. The antibodies RL2 and 110.6 which recognize *O*-GlcNAc on proteins, enable the investigation of multiple *O*-linked glycosylated proteins at once. Specifically, antibody RL2 is an excellent investigative tool for estimating the overall level of *O*-linked protein glycosylation present in the cell. This antibody also provides a reliable estimate of the amount of glucosamine in the cell. Antibody 110.6 also identifies multiple *O*-linked glycosylated proteins, but is a more useful antibody for immunohistochemical investigation of *O*-linked glycosylation.

The study of *N*-linked glycosylation has its own set of challenges. As previously described, the state of *N*-linked glycosylation is specific to each individual protein, and it changes depending on the stage of synthesis, processing and cellular location of a given protein. However, the extent of *N*-linked glycosylation does affect the molecular weight of a protein such that changes in glycosylation can be seen by immunoblot. In order to further investigate the effect of increased cellular glucosamine levels on *N*-linked glycosylation, specific protein targets must be selected for further study. It has been previously shown that glucosamine treatment of HepG2 cells, inhibits the *N*-linked glycosylation of ApoB-100 and α_1-antitypsin (α_1-AT) when studied using radiolabelling and immunoprecipitation[63]. Additionally, glucosamine treatment of RAW 264.7 and ldlA[6] cells showed that the *N*-linked glycosylation of mouse Scavenger Receptor – BI (mSR-BI) is also prevented[64]. To further this study, we investigated the effect of

increased cellular glucosamine levels on the *N*-linked glycosylation of human Scavenger Receptor – BI (hSR-BI) and α_1-AT in HepG2 cells, by immunoblot.

1.8 Scavenger Receptor – BI

SR-BI is a 509 amino acid, cell surface, membrane protein [65]. It has a horseshoe-like membrane topology, with a short N- and C-terminal domain, the latter of which is cysteine-rich [65,66]. SR-BI is a member of the CD36 superfamily of proteins, involved in cholesterol transport across the plasma membrane [65]. It is described as a scavenger receptor, due to its ability to bind multiple ligands, including; LDL, Very Low Density Lipoproteins (VLDL), High Density Lipoproteins (HDL), acetylated LDL, oxidized LDL, anionic phospholipids, apoptotic cells, and Advanced Glycation End-products (AGEs). SR-BI is similar to the LDL receptor (LDLR) in that it enables cells to take up lipid in the form of cholesteryl esters from lipoproteins [65]. However, SR-BI facilitates the transfer of cholesteryl ester from lipoproteins to the plasma membrane in a process known as selective lipid uptake. This process differs from that of LDLR cholesterol uptake, in that endocytosis of the lipoprotein-receptor complex does not occur and lyzosomal degradation of the lipoprotein particle is not required. Selective lipid uptake is a two step process involving the binding of the lipoprotein particle to the receptor, followed by the efficient transfer of lipid to the plasma membrane [66]. In some types of cultured cells, SR-BI has been shown to localize with caveolae – which are known sites of cholesterol uptake on the cell surface [66].

Expression of SR-BI is tissue-specific, however, it is highly expressed in both steroidogenic tissues and liver [65,67]. Of significant importance, is SR-BI's ability to bind to apolipoprotein A-I in HDL [66]. SR-BI is a physiologically relevant HDL receptor associated with reverse cholesterol transport and lipid clearance from HDL in the blood [65]. It enables the transfer of cholesteryl ester and lipid from HDL to hepatocytes in the liver, so that it can be metabolized and cholesterol can be excreted as bile [66]. Reverse cholesterol transport plays an important role in the prevention of atherosclerotic plaque development and cardiovascular disease, by preventing the accumulation of lipid in the artery wall. As such, SR-BI in the liver is considered to be anti-atherogenic and this is supported by the observation that hepatic over-expression of SR-BI reduces atherosclerosis in animals models of atherosclerotic cardiovascular disease [65,66,67].

The predicted mass of SR-BI, based on its primary amino acid sequence, is ~57kDa, however SR-BI has 11 potential sites of *N*-linked glycosylation and its observed mass is ~82kDa due to the addition of multiple *N*-linked glycans. The glycosylation of SR-BI occurs both co- and post-translationally in the ER with the addition of multiple *N*-linked, high mannose oligosaccharides. It is further processed in the Golgi to yield a mature protein containing both complex and high mannose oligosaccharides [68].

SR-BI is an effective glycoprotein target in the study on *N*-linked glycosylation due to its multiple and diversely structured glycans. It is also an interesting target in the study of diabetes. As previously described, SR-BI is a physiologically relevant HDL receptor associated with reverse cholesterol transport and lipid clearance from the blood.

The potential effect of glucosamine on SR-BI glcyan structure and function suggests that hyperglycemic conditions could also affect SR-BI, consequently altering cholesterol metabolism, and affecting the development and progression of atherosclerosis. Interestingly, changes in SR-BI expression have been observed under diabetic conditions in animal models [66].

1.9 α_1-Antitrypsin

α-1-antitrypsin (α_1-AT) is small glycoprotein member of the serpin (serum protease inhibitor) family of proteins. It is actually the principal protease inhibitor found in serum and acts to neutralize proteases that are released from leukocytes and neutrophils during infection [69]. As a vital immune response, proteases are secreted to breakdown extrinsic matter. However, proteases also present a threat to innate tissues in the body. α_1-AT acts as a defence protein which functions to protect tissues in the body from attack by proteolytic enzymes. α_1-AT irreversibly inhibits proteases in a 1:1 manner and has often been called a "suicide protein" as the protease-inhibitor complex must be taken up and degraded by cells of the reticuloendothelial system in order to be removed from the blood. The normal plasma concentration of α_1-AT is ~1g/L and it has a 6 day half-life [70,71]. The concentration of the protein can quadruple, to ~3.5-4g/L in response to general inflammatory stimuli, and is therefore considered an acute-phase reactant [70,71]. α_1-AT is a relatively unspecialized serine protease inhibitor, however, it has a selectivity for elastase. α_1-AT is predominantly produced by the liver, however, monocytes and macrophages can also synthesize the protein. It is 394 amino acids in

length and has a single reactive site at methionine 382 [70]. Interestingly, this is less specific to the active site of proteases, than a valine or alanine residue would be at this position [70]. However, the methionine residue enables the oxidation of α_1-AT, rendering it inactive, and thus provides a mechanism by which its potency can be controlled [70].

The predicted mass of α_1-AT, based on its primary amino acid sequence, is ~47kDa, however, it has 3 sites of *N*-linked glycosylation at amino acids 70, 107, 271 [70]. Consequently, α_1-AT has an observed mass of ~52kDa due to the addition of multiple *N*-linked glycans [70]. The protein is denoted α_1-AT because it is a major component of the α_1 electrophoretic band of human plasma [70,71]. When separated by isoelectric focusing, α_1-AT separates into multiple bands, which have been identified as different glycoforms of the protein [71]. The major glycoform is known as *M*6 [70].

Multiple genetic polymorphisms of α_1-AT that have been identified and these mutant forms give rise to disease states which vary in their degree of α_1-AT deficiency [70]. Genetic deficiency of α_1-AT has long been associated with emphysema, due to an uninhibited breakdown of lung tissue, by elastase, during infection and inflammation [70,71]. Of particular interest is the common *Z* mutant of α_1-AT. This form of α_1-AT has the same half-life and inhibitory capacity as the normal *M* type however, the *Z* mutant accumulates in the endoplasmic reticulum of hepatocytes and is only partially secreted [70]. The *Z* mutant, which accumulates in the liver, has immature carbohydrate sidechains [70]. The glycans are high mannose in nature and have no sialic acid, in contrast to the complex di- and tri-antennary glycans of the mature *M* protein [70,71].

Individuals who produce the *Z* mutant suffer from an α_1-AT deficiency, making them prone to developing emphysema, and they have compromised liver function, often leading to fatal cirrhosis of the liver [70].

Studies of α_1-AT deficiency diseases led to the protease-antiprotease theory [69,72]. This theory suggests that the dynamic balance of proteases and their inhibitors in serum is very sensitive and highly regulated. If an imbalance occurs, it can have serious implications. High levels of inhibitor affect the ability of the body to fight infection, while low levels of inhibitor cause proteases to attack innate tissues in the body [69,72].

α_1-AT and its mutants have been widely used, for many decades, in the study of *N*-linked protein glycosylation, ER lectin binding of glycoproteins and ER-associated protein degradation [73]. However, α_1-AT is also an interesting target in the study of diabetes as well. Multiple clinical studies, investigating serum α_1-AT in diabetic patients, have been performed [69,72]. The studies report that diabetic patients have elevated serum α_1-AT levels in both type I and type II diabetes [69,72]. However, it was also found that the serum trypsin inhibitory capacity (TIC) of α_1-AT from these patients, is reduced [69,72]. The results suggest that, while α_1-AT secretion is increased as a result of diabetes, the functionality of α_1-AT is compomised [69,72]. Currently, there is little evidence to suggest why α_1-AT has reduced activity in diabetic individuals however, some experimental evidence suggests that this could be an effect of glucosamine [74]. As previously described, *Z* mutant of α_1-AT is an immaturely glycosylated protein which accumulates in the ER of hepatocytes [70]. Glucosamine has also been shown to have a

similar effect. Studies in Hep G2 cells have shown that glucosamine produces immaturely glycosylated α_1-AT, however secretion of this immature α_1-AT is not affected [74]. This observation is similar to that seen in plasma from diabetic patients. Hyperglycemic conditions increase cellular levels of glucosamine [59]. It is conceivable that glucosamine production in hepatocytes, while having no effect on α_1-AT secretion, may result in the production of an immature, and inactive form of α_1-AT under hyperglycemic conditions.

Diminished levels of active, circulating α_1-AT in the blood makes tissues susceptible to proteolytic attack as a result of inflammatory stimuli. As the protease-antiprotease theory describes, this can have serious, deleterious effects – one of which is vascular damage. It has been suggested that inhibition of elastase by α_1-AT is atheroprotective [75]. α_1-AT has the potential to inhibit the degradation of elastic fibers in the arterial elastic lamina, thus preventing the loss of vascular tone often seen with atherosclerotic plaque formation [75]. In the Lopid Coronary Angiopathy Trial (LOCAT), progression of atherosclerosis in individuals with α_1-AT deficiencies was studied. Quantitative coronary angiography was used to determine disease progression. It was found that individuals with the *Z* mutation of α_1-AT had an 11.5% greater disease progression than controls [75]. This study suggests that low levels of α_1-AT increases the progression of atherosclerosis [75].

Taken together, the clinical studies and experimental work present an interesting hypothesis. Diabetes mellitus and associated-hyperglycemia, could increase hepatocyte

glucosamine levels, affecting both the activity and/or secretion of α_1-AT, due to altered glycosylation, and thus contribute to the progression of atherosclerosis.

1.10 Objective

The objective of this thesis will be to characterize the role of GFAT, the hexosamine pathway and protein glycosylation in ER stress induction.

Our lab has previously shown that treating cells in culture with glucosamine induces ER stress, shown by the increased expression and upregulation of UPR proteins [5]. Based on evidence showing that over-expression of GFAT in cell culture systems will increase flux of glucose through the hexosamine pathway, producing increased levels of cellular glucosamine, **it is hypothesized that GFAT over-expression will be sufficient to induce an increased ER stress response** [76,77,78].

Hyperglycemic conditions have also been shown to increase the cellular levels of protein *O*-linked glycosylation [59]. Increasing cellular glucosamine levels, increases cellular levels of protein *O*-linked glycosylation as well as induces an ER stress response [79]. **It is hypothesized that inhibiting either *O*-linked glycosylation or the deglycosylation of proteins will have an effect on the induction of an ER stress response.**

Finally, glucosamine may also have an effect on protein *N*-linked glycosylation. **We aim to study the effect of increased cellular glucosamine levels on the *N*-linked glycosylation of glycoproteins, as this may play a contributing role to ER stress induction.**

2.0 Materials and Methods

2.1 Materials and Solutions

All Materials and Suppliers are detailed in Table 1.0

Table 1.0 Reagents and Suppliers

Chemical or Reagent [Abbreviation] (Supplier)
[α-^{32}P]dATP (Perkin Elmer)
[α-^{32}P]dCTP (Perkin Elmer)
0.5M Tris-HCl pH 6.8 (Bio-Rad)
1.5M Tris-HCl pH 8.8 (Bio-Rad)
110.6 Antibody (Covance)
18S RNA (a kind gift of Dr. R. Austin, McMaster University, Hamilton, ON)
231 Media (Cascade Biologicals)
4',6-diamidino-2-phenylindole [DAPI] (Vector Laboratories)
9c (1,2-Dideoxy-2'-propyl-α-D-glucopyranoso-[2,1-d]-α-thiazoline) (a kind gift from Dr. D.J. Vocadlo, Simon Fraser University, Burnaby, BC)
A23187 (Sigma)
Acrodisc Syringe Filters 0.2μm (Pall Corporation)
Acrylamide/Bis (37.5:1) (Bio-Rad)
Agarose (Bioshop)
Alloxan (Sigma)
Alpha-1-Antitrypsin (Boeringer Manheim)
Ammonium Persulfate (BRL Life Technologies)
Bio-Rad D_C Protein Assay (Bio-Rad)
Bovine Serum Albumin [BSA] (Sigma)
Diethyl Pyrocarbonate [DEPC] (Sigma)
Dithiothreitol [DTT] (Sigma)
Dulbecco's Modified Eagle's Medium [DMEM] (Gibco)

Endoglycosidase H [Endo H] (Roche)
ExGen 500 (Fermentas)
Fetal Bovine Serum [FBS] (Invitrogen)
Formaldehyde (ACP)
Fructose (Sigma)
GFAT Antibody (a kind gift from Dr. C. Weigart, University of Tubingen, Germany)
GFAT/GFP Adenovirus (a kind gift of Dr. H. Kaneto, Joslin Diabetes Centre, Boston, MA, USA)
GFP Adenovirus (a kind gift of Dr. H. Kaneto, Joslin Diabetes Centre, Boston, MA, USA)
GFP Antibody [Living Colors Full-Length a.v.] (Clontech)
Glucosamine (Sigma)
Glucose (Sigma)
Glutamine (Sigma)
Glycerol (BDH)
Glycine (Bioshop)
Goat Serum (Vector Laboratories)
GRP78 cDNA fragment (a kind gift of Dr. R. Austin, McMaster University, Hamilton, ON)
HRP-conjugated Goat-Anti-Rabbit Antibody (DAKO Cytomation)
HRP-conugated Goat-Anti-Mouse Antibody (DAKO Cytomation)
KCl (Bioshop)
KDEL Antibody (Stressgen)
KH_2PO_4 (EM Science)
Kodak X-Omat Scientific Imaging Film (Perkin Elmer)
Mannitol (Sigma)
Methanol (Caledon)
Modified Eagle's Medium [MEM] Non-essential Amino Acid Solution (Gibco)
Mouse Alexa Secondary (Molecular Probes)
Na_2KPO_4 (EM Science)
NaCl (Bioshop)
N-Glycosidase F (Roche)
Nitrocellulose (Bio-Rad)
Non-fat dry Milk (Carnation)
pCIS-2 (a kind gift of Dr. M.J. Quon, NIH, Bethesda, MD, USA)
pCIS-GFAT (a kind gift of Dr. M.J. Quon, NIH, Bethesda, MD, USA)
pCIS-GFP (a kind gift of Dr. M.J. Quon, NIH, Bethesda, MD, USA)
peGFP-hSR-BI (a kind gift of Dr. B. Trigatti, McMaster University, Hamilton, ON)

Penicillin/Streptomycin (Invitrogen)
Polynucleotide Kinase(Fermentas)
Protease Inhibitor Cocktail (Roche)
PUGNAc (*O*-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenyl Carbamate) (Toronto Research Chemicals)

All commonly used solutions are detailed in Table 2.0

Table 2.0 Solutions

Solution [Abbreviation]	Recipe
4x SDS Sample Buffer	0.06M Tris 10% Glycerol 2% SDS
BSN	48mM Tris 39mM Glycine 10% SDS 2% Methanol
Phosphate Buffered Saline [PBS]	0.7M NaCl 0.01M KCl 0.05M Na_2KPO_4 7mM KH_2PO_4 pH 7.2
SDS Running Buffer	25mM Tris 196mM Glycine 0.1% SDS
Tris Buffered Saline [TBS]	1.5M NaCl 0.1M Tris pH 7.5
Wet Transfer Buffer	0.02M Tris 0.15M Glycine 10% Methanol

2.2 Methods

2.2.1 Maintenance of Cell Cultures

HepG2 cells were cultered in 1mM glucose DMEM supplemented with 5% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were passaged using 1x trypsin-EDTA.

HASMC were cultured in 5mM glucose, 231 media containing 5% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were passaged using 1x trypsin-EDTA.

HEK 293 cells were cultered in 5mM glucose DMEM supplemented with 5% heat-inactivated FBS, 1% penicillin/streptomycin and 1x MEM non-essential amino acid solution. Cells were passaged using 1x trypsin-EDTA.

In all experiments cells were harvested when they reached a density of approximately $1.3x10^5$ cells/cm^2 of tissue culture plate.

2.3 The role of GFAT in Hyperglycemia-associated ER Stress: Experimental Procedures & Treatment Conditions

2.3.1 *Treatment with Simple Sugars and Amino acids*

Where indicated, media was supplemented with glucose, glucosamine, glutamine, glutamic acid, fructose and mannitol at the given concentrations. Cells were harvested after 24hrs incubation with treatments. All compounds were prepared fresh in culture medium at the time of treatment, and sterilized by filtration before addition to the cell cultures.

Hep G2 cells were transiently transfected using ExGen 500, as per the manufacturers directions. Where indicated, cells were transfected with control pCIS-2, pCIS-GFAT and/or pCIS-GFP with the described amounts of plasmid. 24 hours post-transfection, cells were either harvested or treated with the indicated glucose concentrations for the indicated length of time and then harvested. Glucose supplemented media was prepared as described in 2.3.1.

2.3.3 *Infection of Cells with GFAT/GFP and GFP adenovirus*

GFAT/GFP and GFP adenoviral constructs were used to infect Human Aortic Smooth Muscle Cells. A 100 MOI was used to infect the cells. Each virus was diluted in serum-free media before being added to the culture media of the plated cells. Cells were infected for 2 hours. Media was then changed to serum supplemented 231 media and cells were incubated for 24 hours (to allow for expression of the viral proteins). Subsequently, cells were treated with culture media supplemented with various amounts of glucose for the times indicated. Glucose supplemented media was prepared as described in 2.3.1

2.3.4 *Northern Blot Analysis*

Cells were washed with cold PBS prior to lysis. RNA was isolated from cells using the Qiagen RNeasy total RNA Extraction kit and resuspended in DEPC-treated water. Total RNA isolated from cultured cells was quantified by spectrophotometry (Bio-Rad SmartSpec 3000). RNA (10 μg/lane) was size-fractionated on 2.2 M formaldehyde/1.2%

agarose gels, transferred to Zeta-Probe GT nylon membranes and hybridized using cDNA probes. Specific probes were generated by radiolabeling cDNA fragments of GFAT, and GRP78. The probes were labeled with [α-^{32}P]dCTP using a random primer DNA labeling kit (Roche). Blots were hybridized with the probe, and imaged using a Typhoon 9140 phosphoimaging system. 18S RNA was used as control and probes were prepared by end-labeling 18S RNA with [α-^{32}P]dATP using polynucleotide kinase.

2.3.5 *Western Blot Analysis (Procedure 1)*

Cells were washed with cold PBS prior to lysis. Cells were lysed in 4x SDS Sample Buffer. Protein concentrations in the lysates were determined using the Biorad D_C Protein Assay and BSA as a standard. DTT was added to lysates to a final concentration of 0.05M. Samples were boiled for 10 min., equally loaded based on protein concentration and resolved by SDS-PAGE. (Separating gel: 10% acrylamide [30:1 bisacrylamid], 0.4M Tris-HCl pH 8.8, 0.1% SDS. Stacking gel: 4% acrylamide [30:1 bisacrylamid], 0.13M Tris-HCl pH 6.8, 0.1% SDS). SDS-PAGE was performed in an electrophoresis tank containing SDS running buffer. Resolved samples were transferred onto nitrocellulose using the Bio-Rad Semi-Dry Transfer Apparatus and BSN buffer for 1 hour at 15V (Results 3.1). Blots were Ponceau stained to ensure complete transfer. Blots were then incubated over night at 4°C in 5% non-fat dry milk in TBST (TBS containing 0.01% Tween-20) to block non-specific protein binding sites. Blots were incubated with primary antibodies for 1.5 hours at room temperature with shaking/agitation. Antibodies were used at the following concentrations (KDEL 1:1000, GADD 153 1:100, GFAT

1:1000, β-actin 1:5000) in 1% non-fat dry milk in TBST. Blots were washed 3 times for 10 min. in TBST. Horseradish peroxidase (HRP) conjugated anti-IgG (goat anti-mouse or goat anti-rabbit, where appropriate, at 1:200 dilutions in 1% non-fat dry milk in TBST) were then incubated for 45 min at room temperature with shaking/agitation. Blots were washed an additional 3 times for 10 min. in TBST. Western Lightning Chemiluminescense Reagent was used to detect HRP activity. Blots were then exposed to audoradiography film.

2.3.6 *Immunofluorescence*

HAMSC were plated on coverslips and transfected/infected with GFAT/GFP (as previously described in 2.3.2 and 2.3.3). Cells were washed three times with 1x PBS and fixed for 10min. in cold 1:1 methanol:acetone, on ice. Cells were washed 3 times with PBS and blocked in goat serum for 30min. Primary antibodies (GFAT, 110.6, KDEL - 1/200 dilutions of all antibodies in PBS) were incubated on cells for 2 hours. Cells were washed three time in PBS and incubated for 1 hour in appropriate Alexa secondary antibody. Cells were washed a final three times with PBS, and incubated for 10 minutes with DAPI for nuclei staining. Cells were visualized using a Zeiss Axioscope 2 microscope.

2.4 The role of Protein Glycosylation in Glucosamine-induced ER stress: Experimental Procedures & Treatment Conditions

2.4.1 *Alloxan Treatment*

Hep G2 cells were pre-treated with 20mM alloxan for 24 hours in DMEM. Media was then changed to DMEM supplemented with 20mM alloxan plus tunicamycin (5μg/mL), A23187 (5μM) or 5mM glucosamine, as indicated. As controls, cells which had not been treated with alloxan were treated with tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine for 24 hours. Alloxan and glucosamine were dissolved in DMEM at the time of cell treatment and sterilized by filtration.

2.4.2 *PUGNAc Treatment*

Hep G2 cells were treated with 50 μM *O*-GlcNAcase inhibitor PUGNAc 24 hours in DMEM. Cells were then treated with tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine for 8 hours. As controls, cells which had not been treated with PUGNAc were treated with tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine for 8 hours. Glucosamine was dissolved in DMEM at the time of cell treatment and sterilized by filtration.

2.4.3 *9c Treatment*

Hep G2 cells were treated with 50 μM *O*-GlcNAcase inhibitor 9c 24 hours in DMEM. Cells were then treated with tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine for 8 hours. As controls, cells which had not been treated with 9c were

treated with tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine for 8 hours. Glucosamine was dissolved in DMEM at the time of cell treatment and sterilized by filtration.

2.4.4 *N-linked Glycosylation Experiments*

HepG2 cells were transiently transfected using ExGen 500, as per the manufacturers directions. Cells were transfected for 24 hours with peGFP-hSR-BI (0.1 ug/cm^2 of plated cells). Cells were then treated for a subsequent 24 hours with DMEM that was supplemented with 5mM glucosamine. Glucosamine was dissolved in DMEM at the time of cell treatment and sterilized by filtration.

2.4.5 *Western Blot Analysis (Procedure 2)*

Cells were washed with cold PBS prior to lysis. Cells were lysed in 0.2x PBS containing 0.1% Triton-X 100 and 2x concentration of Protease Inhibitor Cocktail. Cells were frozen on plates at -80°C for 10 min to aid in the lysing process. Lysates were scraped and centrifuged at 3000 rpm for 10 min at 4°C to remove nuclei and cellular debris. Protein concentrations in the lysates were determined using the Bio-Rad D_C Protein Asay and BSA as a standard. Where indicated lysates were treated with N-Glycosidase F (1U / 100ug total protein) and Endoglycosidase F (5 x10^{-5}U / 100ug total protein) for 16 hours at 37°C. 4x SDS Sample Buffer was added to the lysates to a final concentration of 2x. DTT was added to lysates to a final concentration of 0.05M. Samples were boiled for 10 min., equally loaded based on protein concentration and resolved by SDS-PAGE.

(Separating gel: 10% acrylamide [30:1 bisacrylamid], 0.4M Tris-HCl pH 8.8, 0.1% SDS. Stacking gel: 4% acrylamide [30:1 bisacrylamid], 0.13M Tris-HCl pH 6.8, 0.1% SDS). SDS-PAGE was performed in an electrophoresis tank containing SDS running buffer. Resolved samples were transferred onto nitrocellulose using the Bio-Rad Wet Transfer apparatus and transfer buffer for 1.5 hours at 200 mA (Results 3.2) .

Blots were Ponceau stained to ensure complete transfer. Blots were then incubated over night at 4°C in 5% non-fat dry milk in TBST (TBS containing 0.01% Tween-20) to block non-specific protein binding sites. Blots were incubated with primary antibodies for 1.5 hours at room temperature with shaking/agitation. Antibodies were used at the following concentrations (SR-BI 400-101 1:1000, α-1-antitrypsin 1:1000, GFP 1:1000, KDEL 1:1000, GADD 153 1:100, GFAT 1:1000, β-actin 1:5000). SR-BI 400-101, GFP and α-1-antitrypsin antibodies were diluted in TBST alone. All others were diluted in 1% non-fat dry milk in TBST. Blots were washed 3 times for 10 min. in TBST. Horseradish peroxidase (HRP) conjugated anti-IgG (goat anti-mouse or goat anti-rabbit, where appropriate, at 1:200 dilutions) were then incubated for 45 min at room temperature with shaking/agitation. Blots were washed an additional 3 times for 10 min. in TBST. Western Lightning Chemiluminescense Reagent was used to detect HRP activity. Blots were then exposed to audoradiography film.

2.5 Image and Statistical Analysis

All Northern and Western blot results were quantified using ImageQuant 5.2 software. Data was analyzed using Microsoft Excel and the student's *t*-test was considered significant when $p < 0.05$.

3.0 Results

3.1 The role of GFAT in Hyperglycemia-associated ER Stress

3.1.1 Examining the Effect of Simple Sugars and Amino Acids on GFAT activity in HepG2 Cells

Glucosamine has long been a recognized ER stress inducing agent [39]. Our lab has previously shown that treatment of HepG2 cells with glucosamine or high concentrations of glucose results in increased mRNA and protein expression of GRP78 and GADD153 – two protein markers of ER stress and UPR induction [4].

GFAT, the rate-limiting enzyme in the conversion of glucose to glucosamine, has been shown to be highly regulated by its substrates and products [35,38,80]. Consequently, we examined the effect of substrates and products of GFAT enzyme activity on ER stress markers in HepG2 cells (Figure 5.0). Glucose, glucosamine, glutamine, glutamic acid, fructose, and a mannitol hyperosmotic stress control were used in various concentrations to treat Hep G2 cells for 24 hours. The level GRP78 expression was not affected by any of the treatments except glucosamine and glutamic acid. Treatment with 5, 10 and 30mM glucosamine induced increased GRP78 protein levels.

Glutamic acid treatment was lethal to the cells, resulting in decreasing levels of total protein (as evidenced with β-Actin loading control) as the concentration of the treatment increased. This may be a result of glutamate toxicity, which is a common

cellular phenomenon as glutamate is a potent neurotransmitter [81]. It is also possible that glutamic acid treatment perturbed the pH of the culture media and affected cell growth, though this was not evaluated.

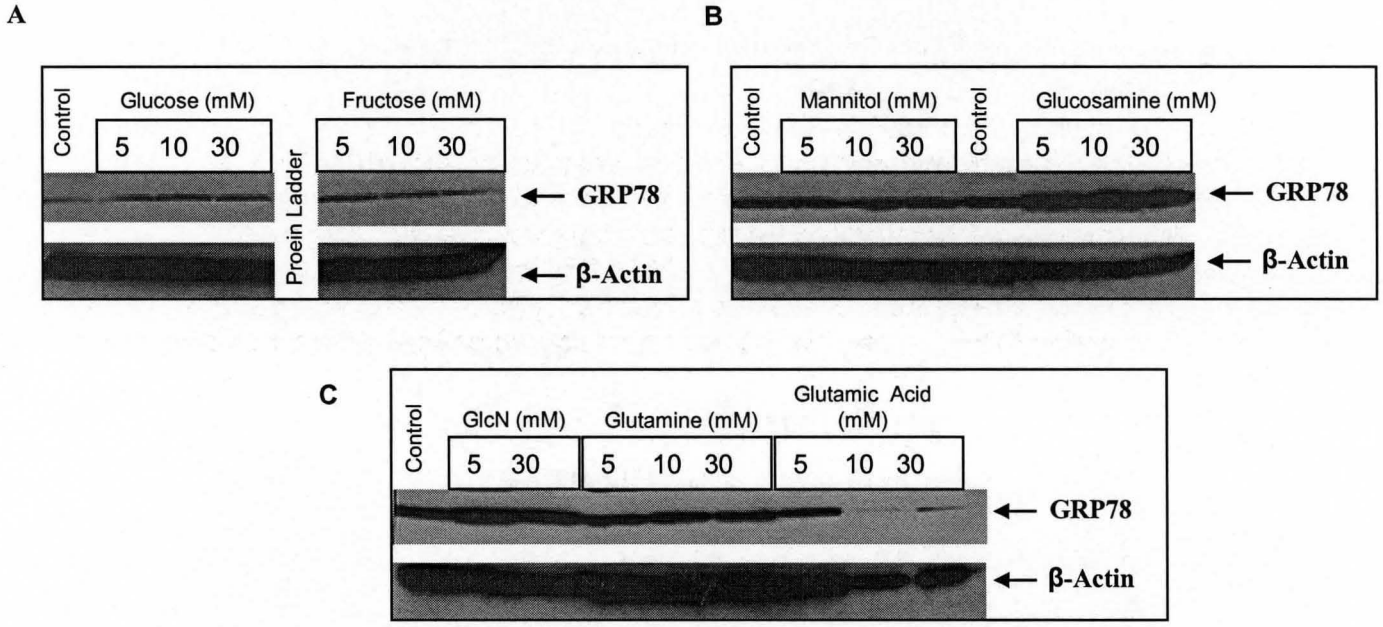


Figure 5.0 Western Blot Analysis of Hep G2 Cells Treated with Substrates and Products of GFAT enzyme activity. Hep G2 cells were treated with 5, 10, and 30mM glucose, fructose, glutamine, glutamic acid, glucosamine. Mannitol and 1mM glucose (control) were used as control conditions. Immunoblotting with anti-KDEL antibody for GRP78 (ER stress marker) is shown. **(A&B)** Glucose, fructose, and mannitol treatments had no apparent effect on protein expression. Treatment with glucosamine increased expression of GRP78. **(C)** Glutamine treatment had effect on expression of GRP78 compared to control. Glutamic acid is toxic to cells, as seen by decreased expression of GRP78 and β-actin compared to control. β-actin was used as a protein loading control. **Note:** Cell density was ~ 1.3×10^5 cells/cm^2.

3.1.2 The Effect of GFAT over-expression and Hyperglycemic Conditions on ER Stress induction and protein *O*-linked glycosylation levels in HepG2 cells

It has been shown that glucose flux through the hexosamine pathway is increased under hyperglycemic conditions, producing high levels of glucosamine [2,4]. Furthermore, we have observed a correlation between elevated levels of *O*-linked glycosylation and ER stress in a hyperglycemic mouse models [4]. Given that glucosamine is used in the glycosylation of proteins and is a recognized ER stress inducing agent, GFAT was over-expressed in HepG2 cells to investigate the role of this enzyme in ER stress response and protein glycosylation [39,57,61].

pCIS-2, pCIS-GFAT and pCIS-GFP constructs were kindly donated by Dr. M.J. Quon, NIH, Bethesda, MD, USA. The pCIS-GFAT construct was sequenced by the Institute for Molecular Biology and Biotechnology Laboratory (MOBIXLab) at McMaster University to ensure its integrity. The construct was free from mutation as compared to the previously published sequence (GenBank accession # M90516) [3].

HepG2 cells were transiently transfected with the pCIS constructs and conditions were optimized. Over-expression of GFAT was seen at the mRNA and protein level. 0.15-0.2μg DNA/cm^2 plated cells was observed to be the optimal DNA concentration for transient GFAT expression (Figure 6.0). The level of GFAT mRNA expression was not dependent on the amount of DNA transfected. This is likely the result of the conditions required for ExGen500 transfection. The transfection is based on the "number of equivolents" of ExGen500 to μg of DNA. There is a limit to the amount of ExGen500 which can be used, which is based on the area of plated cells. This limit prevents cell death and growth arrest during the transfection. Consequently, there is a limit to the

amount of DNA that can be effectively transfected, given that there is a specific and limiting amount of ExGen500 that can be used. The threshold amount of DNA can be quickly reached – evident by the lack of dose response seen in the experiment. Additionally, low amounts of DNA transfect less efficiently. This is seen with the 0.1μg DNA/cm^2 plated cells transfection, seen in Figure 6.0, where the transfection was ineffective.

In the experiment, higher concentrations of DNA did have a slight effect on the transfection. High concentrations resulted in an apparent increase in mRNA degradation (smearing is evident at higher DNA concentrations on the Northern blot). Additionally, higher DNA concentrations often resulted in variable and inefficient transfection levels and affected cell viability. Variable transfection efficiency can often occur when high concentrations of DNA are used. This may result from the depletion of transcription factors that can be sequestered by a highly active promoter of the transfected vector. This can affect overall gene transcription and consequently protein expression in the cell. The optimal concentration of DNA was chosen (0.15-0.2ug DNA/cm^2 plated cells), allowed for over-expression without affecting cell survival. Cell density was ~ 1.3x10^5 cells/cm^2.

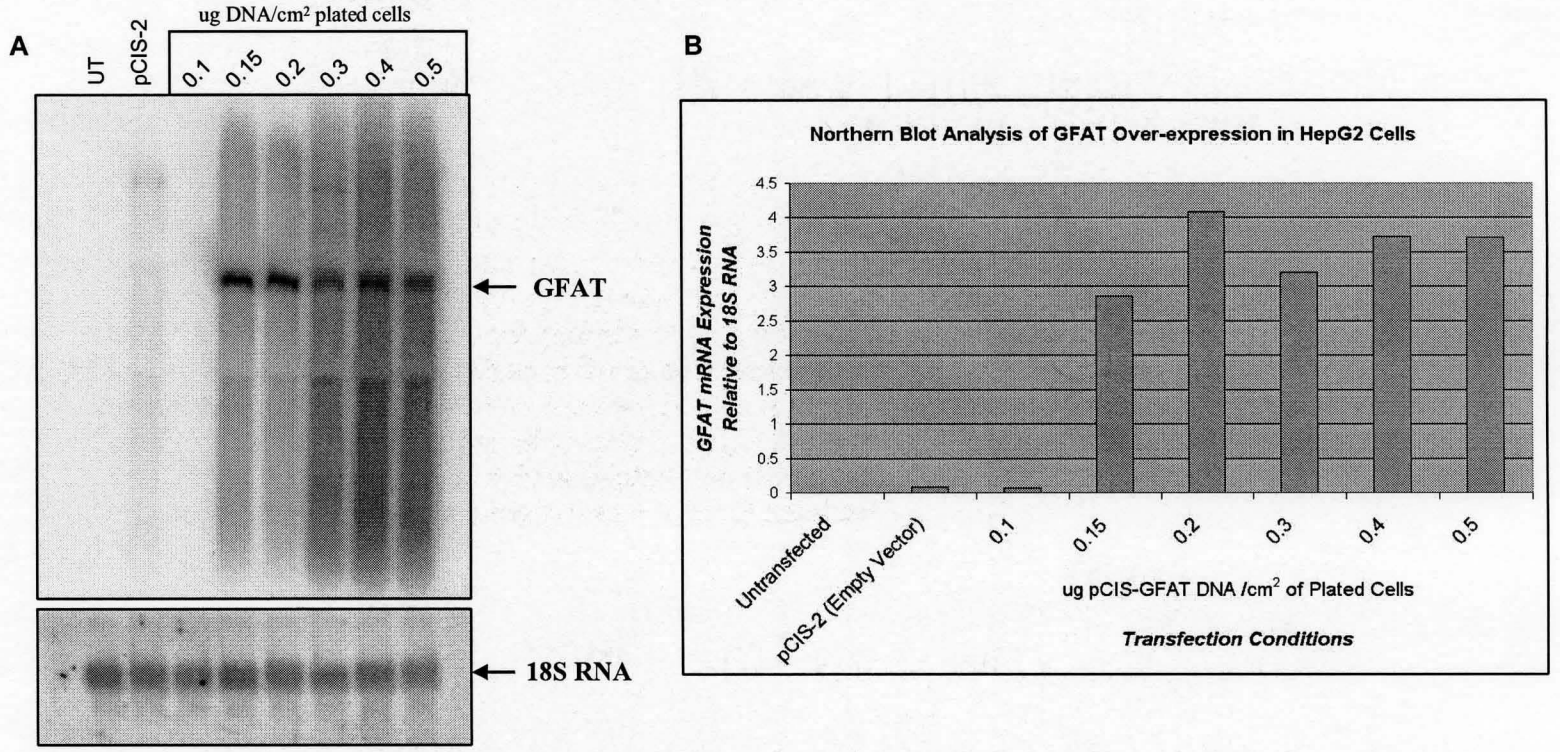


Figure 6.0 Northern Blot Analysis of GFAT Over-expression in HepG2 Cells.
(A) Total RNA was isolated from Hep G2 cells (untransfected) and Hep G2 cells transiently transfected with pCIS-2 (0.3ug pCIS-2/cm² plated cells) and pCIS-GFAT (0.1ug cDNA/cm² plated cells to 0.5ug cDNA/cm² plated cells). **(B)** Phosphoimaging results and ImageQuant software were used to quantify the band intensity seen on Northern Blot in (A). Band intensities of GFAT were normalized to the band intensity of the 18S RNA loading control. The results show that expression of GFAT mRNA in pCIS-GFAT transfected Hep G2 cells is significantly elevated compared to untransfected (UT) and pCIS-2 controls. Transfections of roughly 0.15ug – 0.2ug pCIS-GFAT/cm² plated cells are optimal, having high GFAT expression and a lower level of mRNA degradation (smearing). **Note:** Each lane (A) and bar on the graph (B) represents a single transfection. Cell density was ~ 1.3×10^5 cells/cm².

HepG2 cells were transiently transfected for 24 hours, and subsequently treated with 5 or 30mM glucose for 8 hours. As positive controls, cells were treated with tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine for 8 hours. Northern and Western blotting were used to determine any increase in ER stress marker expression. Western blot analysis was additionally used to monitor any changes in protein *O*-linked glycosylation.

Northern blot analysis (Figure 7.0) shows a ~ 600-900 fold increase in GFAT mRNA expression in Hep G2 cells transiently transfected with pCIS-GFAT plasmid compared to control. Induction of ER stress was monitored using GRP78 expression levels as a marker for induced UPR. GFAT over-expression did not result in increased levels of GRP78 mRNA compared to control. The results also show that GRP78 expression is increased 20-35 fold by treatment of cells with ER-stress inducing agents; tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine, however, GFAT over-expression is not sufficient to induce as similar response.

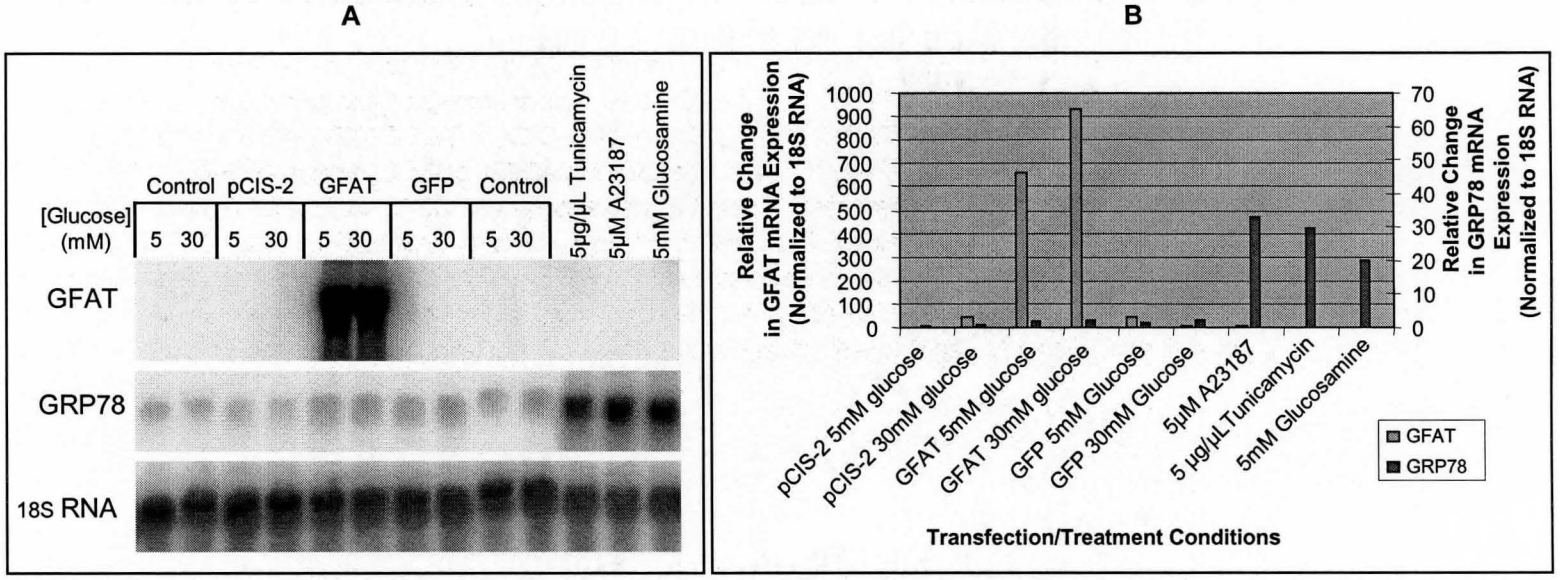


Figure 7.0 Northern Blot Analysis of ER Stress in GFAT transfected Hep G2 Cells.
Hep G2 cells were transiently co-transfected with pCIS-2 + pCIS-GFP (0.15ug pCIS-2/cm^2 plated cells +0.05ug cDNA/cm^2 pCIS-GFP) pCIS-GFAT + pCIS-GFP (0.15ug pCIS-GFAT/cm^2 plated cells + 0.05ug pCIS-GFP/cm^2 plated cells) and pCIS-GFP alone (0.15ug cDNA/cm^2 plated cells). Transfections were carried out for 24 hours and cells were then treated with 5mM or 30 mM glucose for 8 hours. Untransfected cells were treated with ER stress inducers 5µM A23187, 5µg/µL tunicamycin and 5mM glucosamine for 8 hours. **A)** Is a representive Northern blot. **B)** The quantified, fold change in both GFAT and GRP 78 expression shown in A). Results were normalized to 18S RNA loading control and fold increase was determined relative to the untransfected control. **Note:** This experiment is a representative of one of four separately repeated experiments. Each lane (A) and bar on the graph (B) represents a single sample. Cell density was ~ 1.3x10^5 cells/cm^2.

The aim of the subsequent experiment was to determine if the increase in GFAT mRNA levels corresponded to increased GFAT protein expression (Figure 8.0). Western blot analysis shows a ~2 fold increase in GFAT protein levels in HepG2 cells transiently transfected with pCIS-GFAT plasmid compared to control. Immunoblotting with anti-KDEL (for GRP78) did not show an increase in the protein levels of the ER stress marker as a result of GFAT over-expression. This is consistent with the results from the Northern Blot experiment (Figure 7.0) GRP78 protein levels were increased ~2-8 fold in cells treated with ER-stress inducing agents; tunicamycin (5μg/mL), A23187 (5μM), and 5mM glucosamine

Previous studies have shown that hyperglycemia correlates with increased protein glycosylation [59,79]. Monitoring cellular *O*-linked glycosylation levels is a commonly used method to estimate cellular levels of glucosamine, as well as glucosamine produced by over-expression of GFAT [79,77]. HepG2 cells treated with hyperglycemic glucose concentrations (30mM) had elevated levels of *O*-linked glycosylation compared to 5mM glucose treated controls, as seen by immunoblotting with antibody RL2. Previous studies have shown that an increase in GFAT expression, and hexosamine pathway flux, is reflected in the level of protein *O*-linked glycosylation [79,77]. However, GFAT over-expression did not have an appreciable effect on protein glycosylation compared to controls. The results suggest that GFAT protein expression does not significantly increase cellular levels of glucosamine in HepG2 cells. This result most likely explains the lack of induced ER stress response by GFAT over-expression.

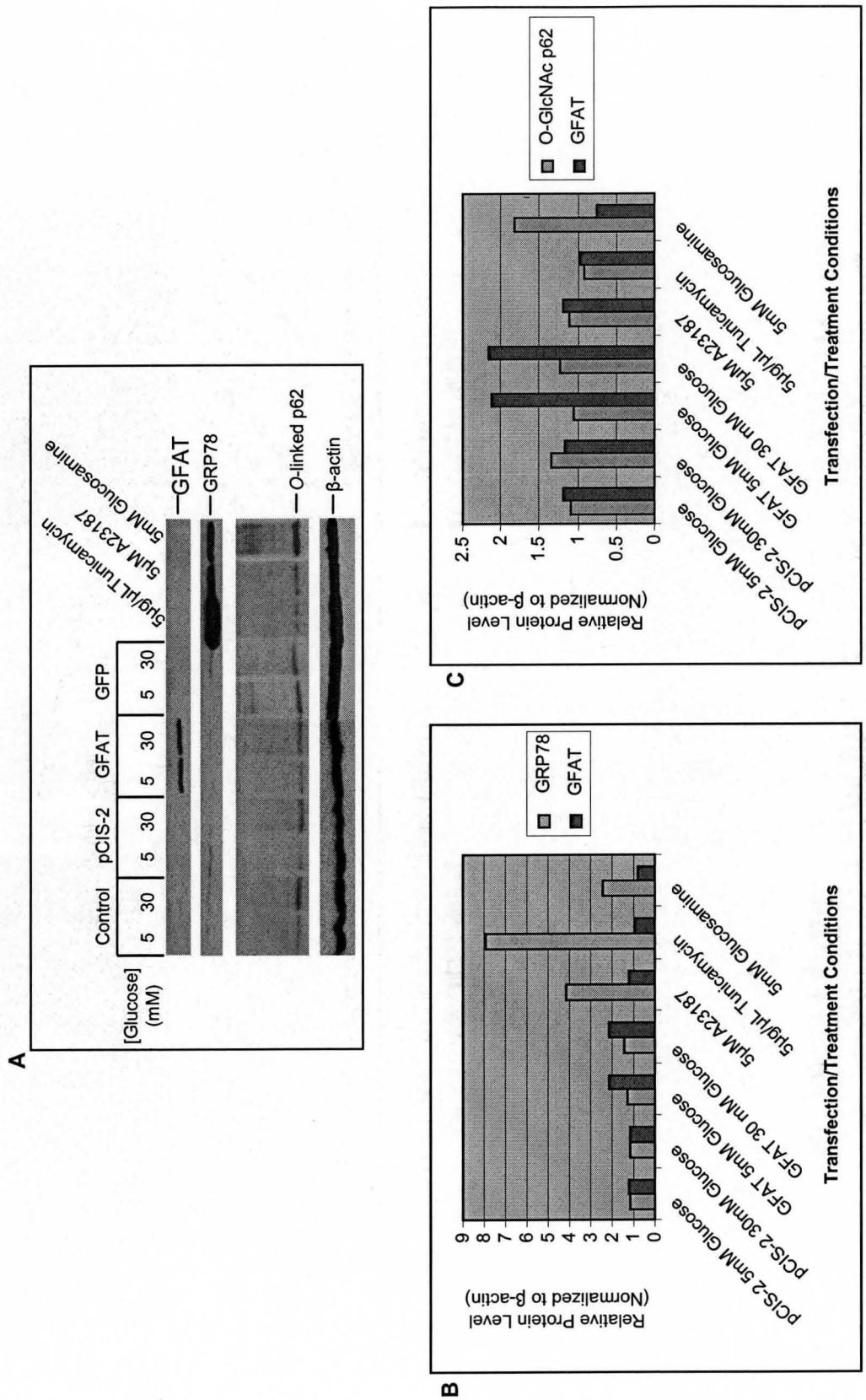

A
[Glucose] (mM)
Control
pCIS-2
GFAT
GFP
5 30 5 30 5 30 5 30
5μg/μL Tunicamycin
5μM A23187
5mM Glucosamine
GFAT
GRP78
O-linked p62
β-actin
B
Relative Protein Level (Normalized to β-actin)
GRP78
GFAT
pCIS-2 5mM Glucose
pCIS-2 30mM Glucose
GFAT 5mM Glucose
GFAT 30 mM Glucose
5μM A23187
5μg/μL Tunicamycin
5mM Glucosamine
Transfection/Treatment Conditions
C
Relative Protein Level (Normalized to β-actin)
O-GlcNAc p62
GFAT
pCIS-2 5mM Glucose
pCIS-2 30mM Glucose
GFAT 5mM Glucose
GFAT 30 mM Glucose
5μM A23187
5μg/μL Tunicamycin
5mM Glucosamine
Transfection/Treatment Conditions

Figure 8.0 Western Blot Analysis of ER Stress and Protein Glycosylation in GFAT transfected Hep G2 Cells. Hep G2 cells were transiently co-transfected with pCIS-2 + pCIS-GFP (0.15ug pCIS-2/cm^2 plated cells +0.05ug cDNA/cm^2 pCIS-GFP) pCIS-GFAT + pCIS-GFP (0.15ug pCIS-GFAT/cm^2 plated cells + 0.05ug pCIS-GFP/cm^2 plated cells) and pCIS-GFP alone (0.15ug cDNA/cm^2 plated cells). Transfections were carried out for 24 hours and cells were then treated with 5mM or 30 mM glucose for 18 hours. Untransfected cells were treated with ER stress inducers 5μM A23187, 5μg/μL tunicamycin and 5mM glucosamine for 18 hours. **A)** Is a representive of immunoblots from a multiply replicated experiment. The quantified results of A) for GFAT, GRP 78 and *O*-linked p62 expression are shown in **B)** and **C)**. Results were normalized to a β-actin loading control and fold increase was determined relative to an untransfected control. Immunoblotting for both experiments was performed with anti-GFAT, anti-KDEL, RL2 and anti-β-actin antibodies. **Note:** This experiment is a representative of one of four separately repeated experiments. Each lane (A) and bar on the graphs (B) and (C) represents a single sample. Cell density was ~1.3x10^5 cells/cm^2

The Northern and Western blot results are significant as they show that GFAT protein over-expression is achieved with transient transfection. It should be noted, however, that analysis of *O*-linked glycosylation is an indirect measure of GFAT activity [79,77].

From these results, we could not draw any major conclusions as to the potential role of GFAT in glucosamine production and ER stress induction under hyperglycemic conditions. What is interesting to note from these studies is the glucose/glucosamine sensitivity of HepG2 cells. In all studies, the ER stress response in the cell line was not sensitive to hyperglycemic (30mM glucose) conditions. To further our studies into the role of GFAT, we investigated alternate cell types, as potential model systems for our GFAT over-expression experiments. Prior to these further experiments, it was essential that the glucose and glucosamine sensitivity in these cell lines be characterized, in order to determine if they would be ideal experimental models.

3.1.3 Glucose and Glucosamine Sensitivity Studies in Human Aortic Smooth Muscle Cells (HASMC) and Human Embryonic Kidney (HEK) 293 Cells

Western blotting for GRP78 and GADD 153 was used to diagnose conditions of ER stress in cells exposed to increasing concentrations of glucose and glucosamine. As well, cellular levels of glucosamine were monitored through study of *O*-linked glycosylation.

3.1.3.1 Glucose and Glucosamine Treatment of Human Aortic Smooth Muscle Cells

HASMC were treated for 4, 8, 16 and 24 hours with increasing concentrations of glucose from 5mM (control) to 70mM. Western blot analysis using KDEL antibody shows that HASMC are sensitive to glucose treatment (Figure 9.0A). While results are variable, there is a slight dose dependent increase in GRP78 expression. The lysates were also immunoblotted for GADD 153, but this ER stress marker was undetectable. This is consistent with previous studies in our lab indicating that HASMC have a low cellular level of GADD 153.

Immunoblotting with antibody RL2 showed that the level of *O*-linked glycosylation varied in slight proportion to the concentration of glucose supplied to the cells (Figure 9.0B). This suggested that high glucose concentrations increase cellular glucosamine levels in HASMC. Additionally, mannitol was used as a hyperosmotic stress control and results showed that high concentrations of mannitol had no affect on ER stress response or *O*-linked glycosylation.

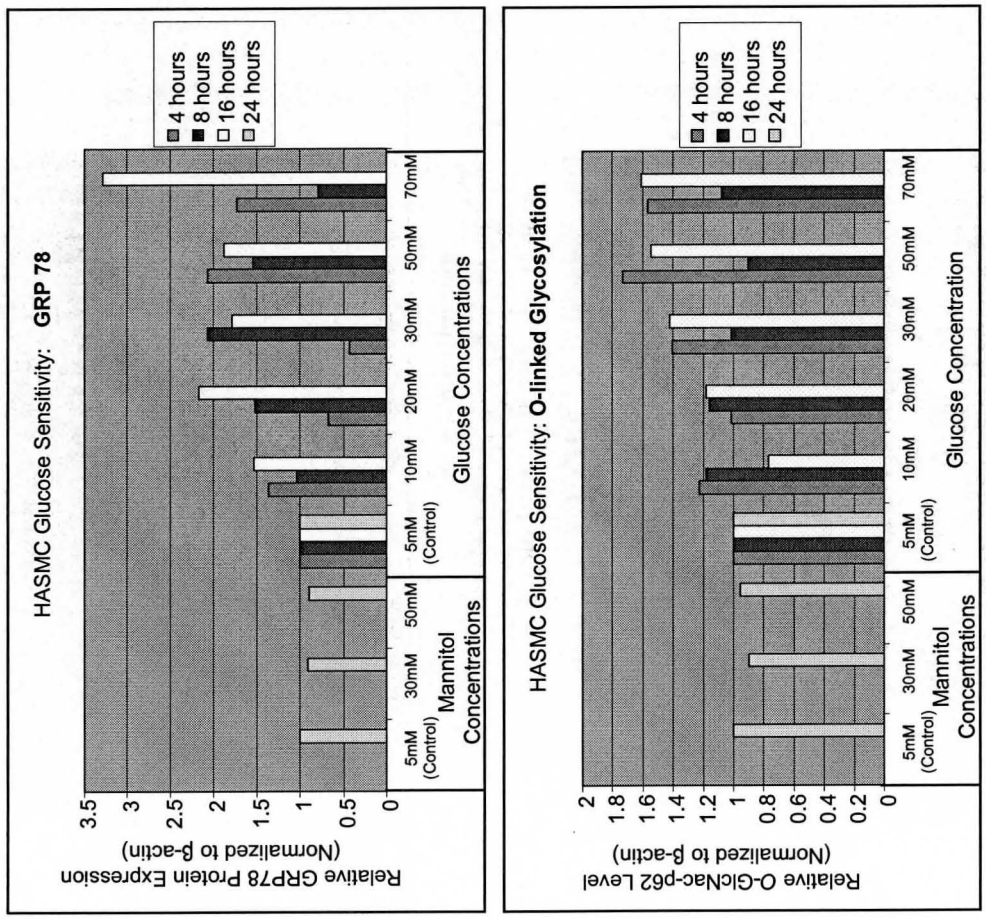


Figure 9.0 Glucose Sensitivity in HASMC. HASMC were treated for 4,8,16 and 24 hours with 5mM (control) - 70mM concentrations of glucose as well as a 24 hour treatment with 30 and 50mM mannitol as an osmotic stress control. Protein expression was determined by Western Blot and quantified. All results were normalised to a β-actin loading control and fold change was determined relative to the 5mM control. **A)** GRP 78 expression was determined using KDEL antibody. **B)** O-linked glycosylation was determined using RL2 antibody. 24 hour treatment of cells with 10mM-70mM glucose caused cell death and are shown as 0 values. **Note:** Each bar on the graphs, (A) and (B), represents a single sample. Cell density was ~1.3x10^5 cells/cm^2 where cell death did not occur.

HASMC were treated for 4, 8, 16 and 24 hours with increasing concentrations of glucosamine from 0mM (control) to 5mM. Western blot analysis using KDEL antibody shows that HASMC are sensitive to glucosamine treatment (Figure 10.0A) in both a time and dose dependent manner. The lysates were also immunoblotted for GADD 153, but this ER stress marker was again undetectable in this cell type (Figure 11.0). Immunoblotting with antibody RL2 showed that the level of *O*-linked glycosylation was also sensitive to glucosamine in a slight time and dose dependent manner, which is expected given that glucosamine is the substrate for *O*-linked glycosylation (Figure 10.0B). These experiments suggest that HASMC are sensitive to glucose and glucosamine and will be a model system for further GFAT over-expression studies.

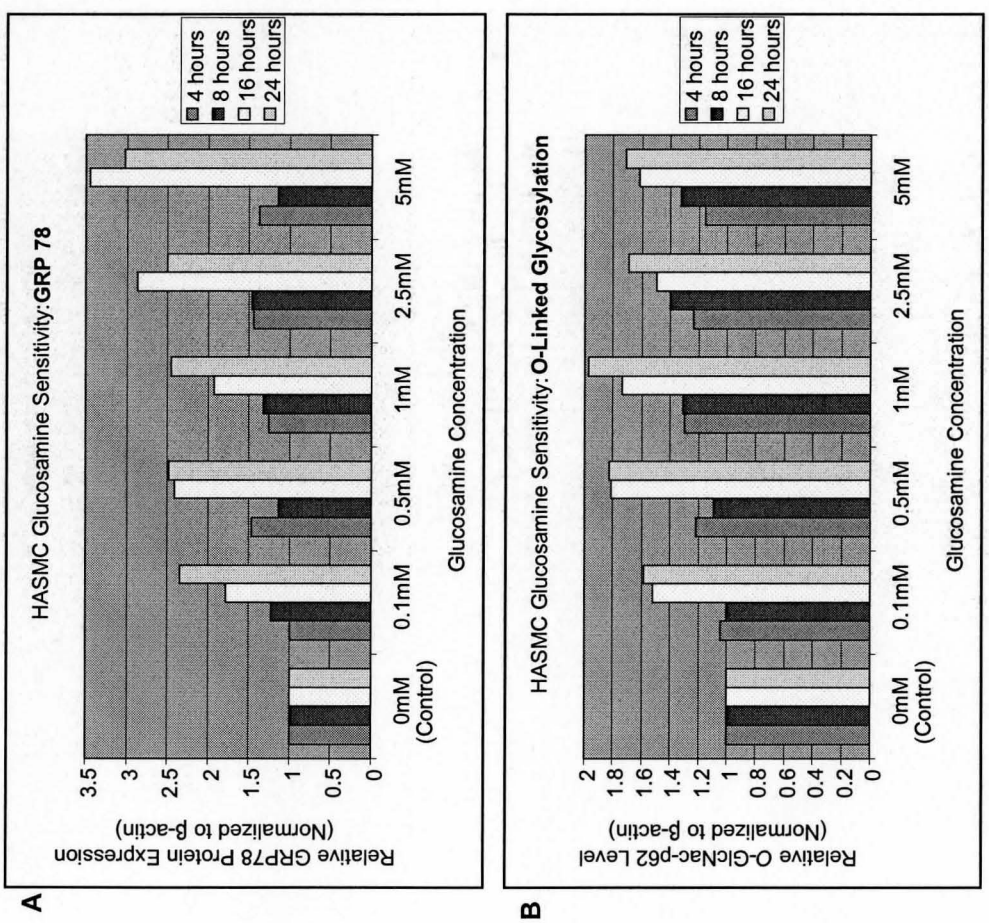


Figure 10.0 Glucosamine Sensitivity in HASMC. HASMC were treated for 4,8,16 and 24 hours with 0mM (control) - 5mM concentrations of glucosamine. Protein expression was determined by Western Blot and quantified. All results were normalised to a β-actin loading control and fold change was determined relative to the 0mM control. **A)** GRP 78 expression was determined using KDEL antibody. **B)** O-linked Glycosylation was determined using RL2 antibody. **Note:** Each bar on the graphs, (A) and (B), represents a single sample. Cell density was ~$1.3x10^5$ cells/cm^2.

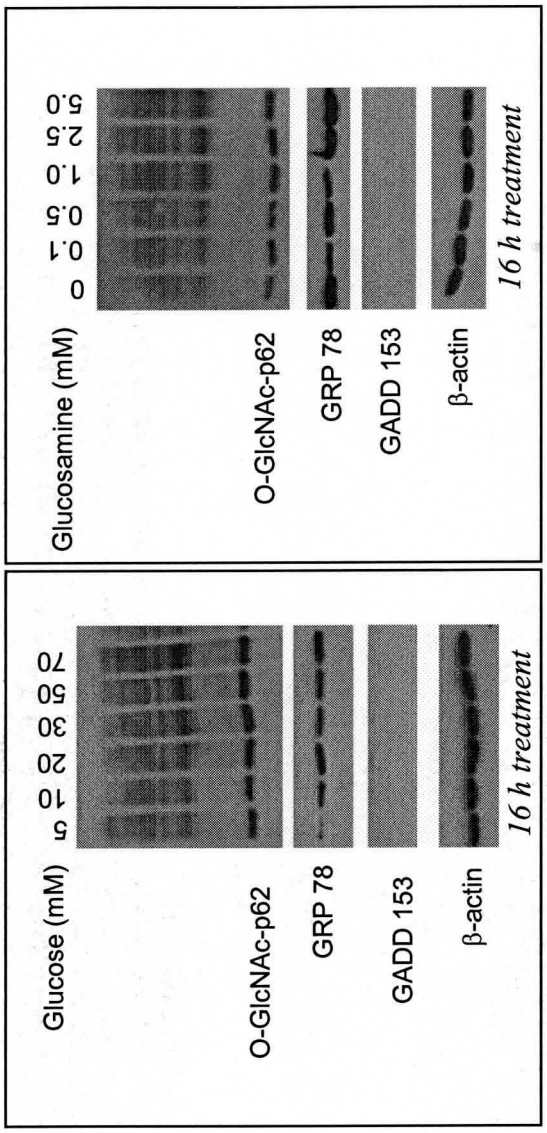
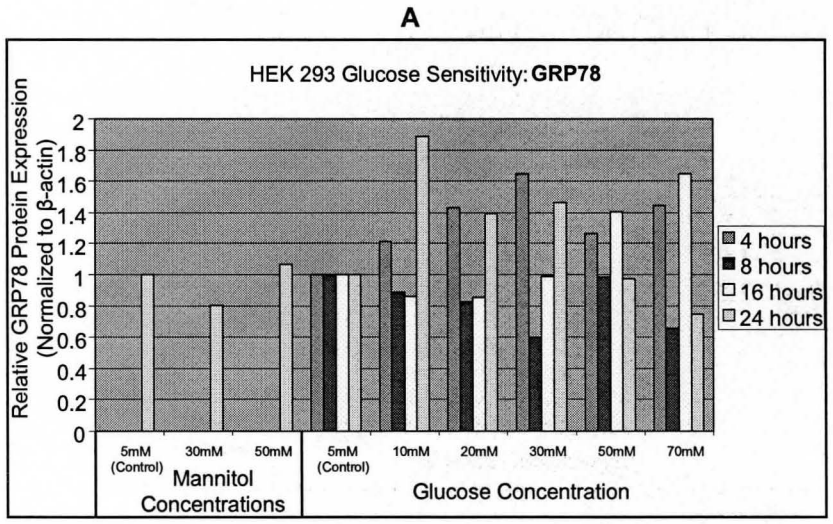


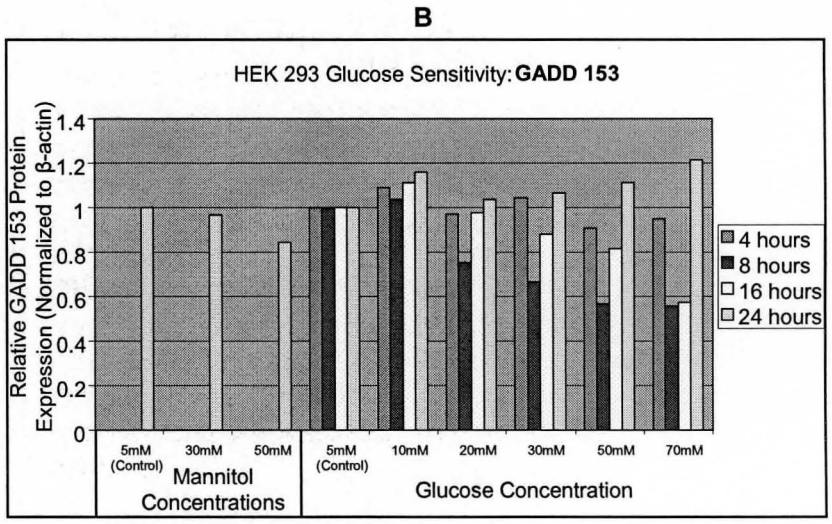
Figure 11.0 16 hour Glucose and Glucosamine Sensitivity in HASMC. These blots are representative of Westerns generated in HASMC treatment experiments. They represent one time point at 16 hours for glucose treatments of 0-70mM and glucosamine treatments of 0mM-5mM. Note that GADD 153 expression can not be seen for this experiment and is typical of previous studies by our lab, in this cell type, indicating HAMSC have a low cellular level of GADD 153.

3.1.3.2 Glucose and Glucosamine Treatment of Human Embryonic Kidney Cells

HEK 293 cells were treated for 4, 8, 16 and 24 hours with increasing concentrations of glucose from 5mM (control) to 70mM. Western blot analysis using KDEL antibody shows that HEK 293 cells are slightly sensitive to glucose treatment, however, the results are highly variable and there was no obvious time or dose dependent trend (Figure 12.0A). The lysates were also immunoblotted for GADD 153 (Figure 12.0B). While GADD 153 expression was detectable in HEK 293 cells, hyperglycemia (30mM glucose) had no effect on inducing its expression above basal levels.

Immunoblotting with antibody RL2 showed that the level of *O*-linked glycosylation was also insensitive to the concentration of glucose supplied to the cells (Figure 12.0C). Again, mannitol was again used as a hyperosmotic stress control and results showed that high concentrations of mannitol had no affect on ER stress response or *O*-linked glycosylation.





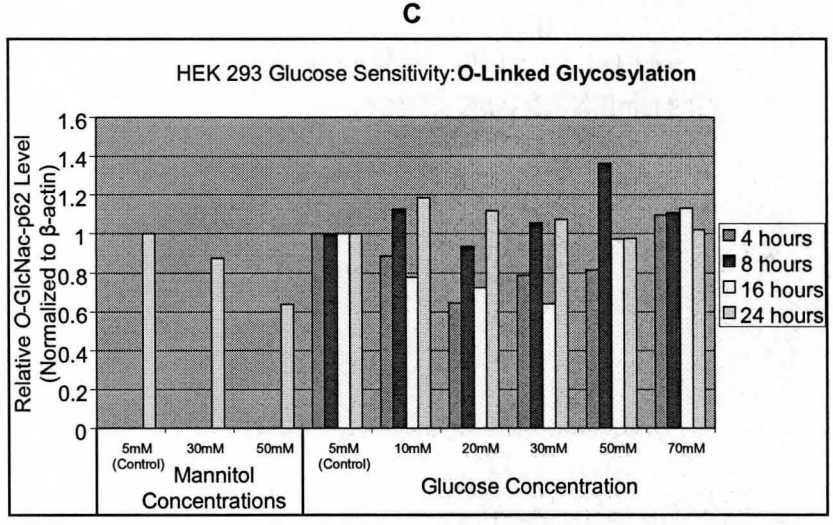


Figure 12.0 Glucose Sensitivity in HEK 293 Cells. HEK 293 cells were treated for 4,8,16 and 24 hours with 5mM (control) - 70mM concentrations of glucose. Protein expression was determined by Western Blot and quantified. All results were normalized to a β-actin loading control and fold change was determined relative to the 5mM control. **A)** GRP 78 expression was determined using KDEL antibody. **B)** GADD 153 expression was deteremined using anti-GADD 153. **C)** O-linked Glycosylation was determined using RL2 antibody.**Note:** Each bar on the graphs, (A), (B) and (C), represents a single sample. Cell density was ~1.3×10^5 cells/cm^2.

HEK 293 cells were treated for 4, 8, 16 and 24 hours with increasing concentrations of glucosamine from 0mM (control) to 5mM (Figures 13.0 and 14.0). Immunoblotting for GRP78 and GADD 153 showed that HEK 293 cells are insensitive to glucosamine treatment (Figures 13.0A and 13.0B). Additionally, the level of *O*-linked glycosylation was not affected by glucosamine treatment in these cells (Figure 13.0C). These experiments suggest that HEK 293 cells are not an ideal system for further GFAT over-expression studies as they appear to be insensitive to both glucosamine treatment and hyperglycemic conditions.

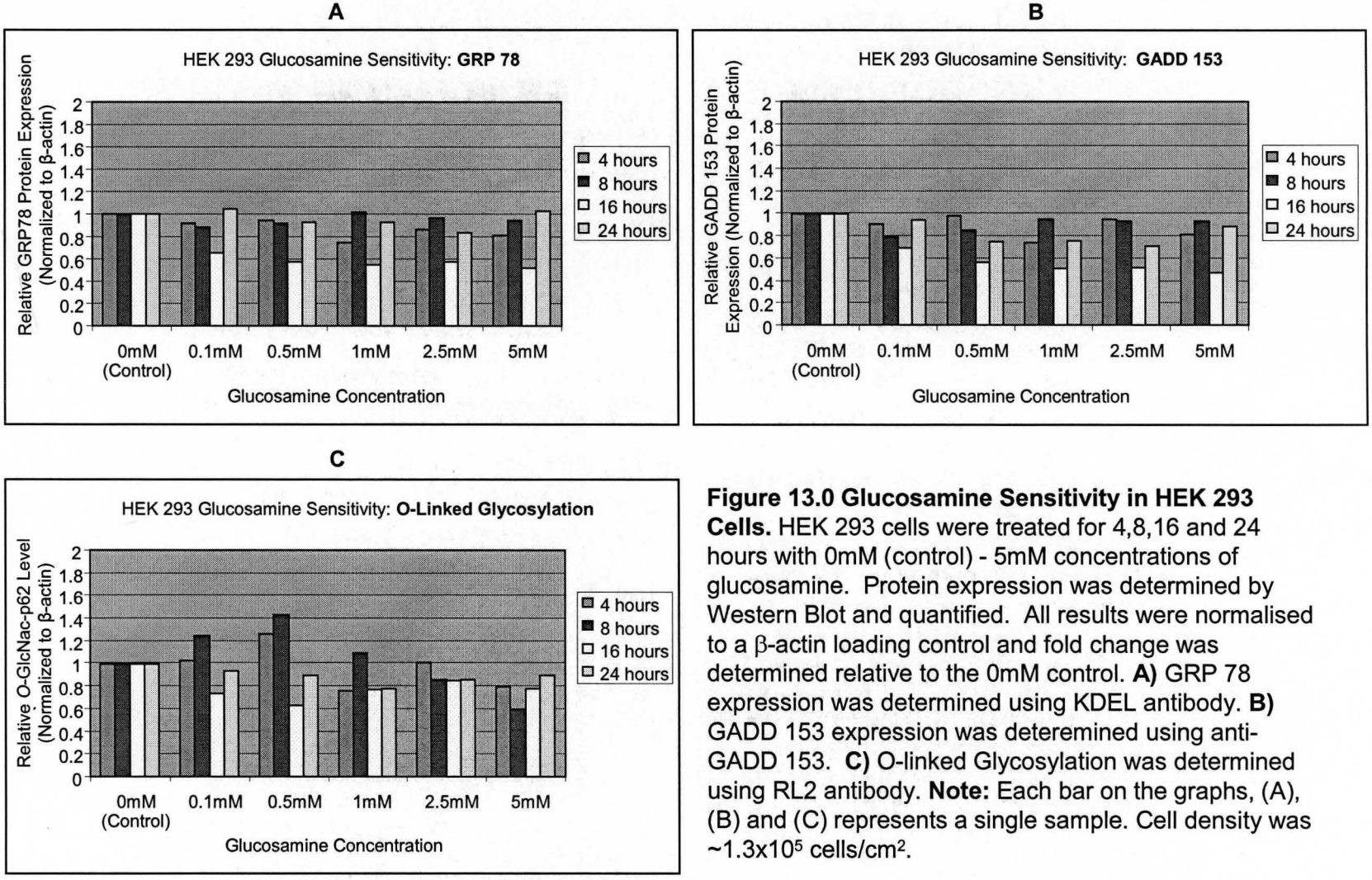


Figure 13.0 Glucosamine Sensitivity in HEK 293 Cells. HEK 293 cells were treated for 4,8,16 and 24 hours with 0mM (control) - 5mM concentrations of glucosamine. Protein expression was determined by Western Blot and quantified. All results were normalised to a β-actin loading control and fold change was determined relative to the 0mM control. **A)** GRP 78 expression was determined using KDEL antibody. **B)** GADD 153 expression was deteremined using anti-GADD 153. **C)** O-linked Glycosylation was determined using RL2 antibody. **Note:** Each bar on the graphs, (A), (B) and (C) represents a single sample. Cell density was ~1.3×10^5 cells/cm^2.

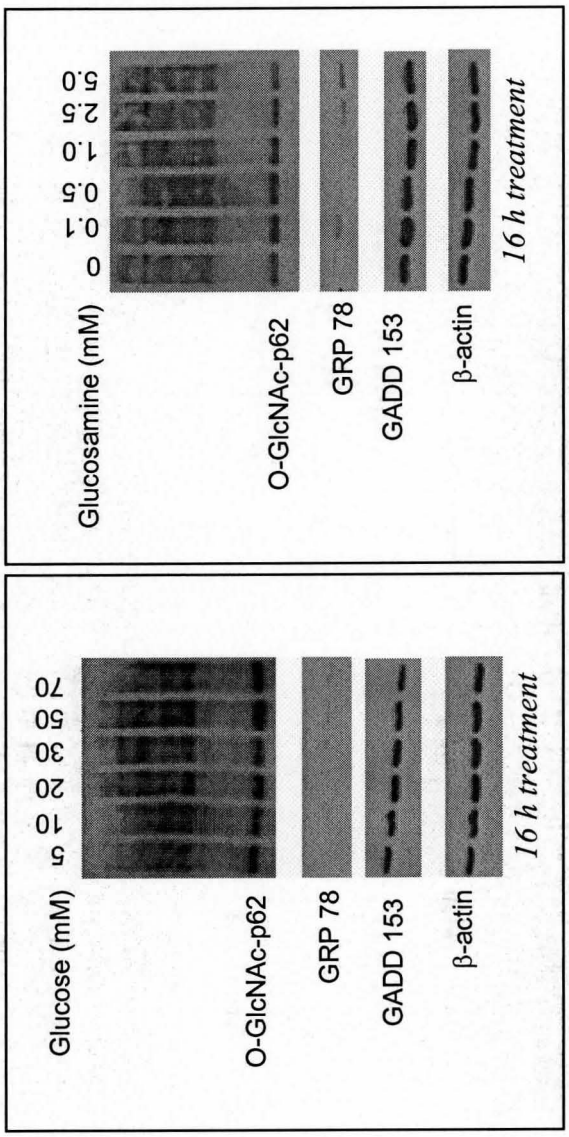


Figure 14.0 16 hour Glucose and Glucosamine Sensitivity in HEK 293 Cells. These blots are representative of Westerns generated in HEK 293 cell treatment experiments. They represent one time point at 16 hours for glucose treatments of 0-70mM and glucosamine treatments of 0mM-5mM.

3.1.4 The Effect of GFAT over-expression on ER Stress induction and protein *O*-linked glycosylation levels in HASMC

Based on the results of the sensitivity studies (section 3.1.3), GFAT over-expression was attempted in HASMC using transient transfection of the previously described pCIS constructs. In addition, a GFAT adenovirus construct, donated by Dr. H Kaneto, Joslin Diabetes Centre, Boston, MA, USA was used to infect the cell line (see Materials and Methods section 2.3.3). The ability of GFAT to induce ER stress or affect protein glycosylation was investigated, similar to experiments in section 3.1.2.

Initial attempts to over-express GFAT in HASMC were met by a number of set backs. Transfection using pCIS constructs caused a considerable amount of growth arrest and cell death in HASMC. Our initial conclusion was that the transfection reagents were causing this problem and subsequently investigated the effect of treating the HASMC with each component of the transfection. This had no effect on cell survival. We concluded that the amount of DNA being used to transfect the cells may be too high and consequently killing off transfected cells. The over-expression of GFAT in HASMC using both pCIS-GFAT and GFAT adenovirus constructs were optimized for conditions that increase GFAT protein levels, without causing cell death – particularly in pCIS-GFAT transfected cells. The ability of GFAT to induce ER stress or affect *O*-linked protein glycosylation in these experiments was studied by immunoblot. Figure 15.0 shows an increase in GFAT protein levels in HASMC cells transiently transfected with pCIS-GFAT plasmid or infected with GFAT adenovirus, compared to control. Immunoblotting with antibody RL2 did not show an increase in protein *O*-linked

glycosylation as a result of GFAT over-expression –indicating that cellular levels of glucosamine were not increased as a result of GFAT over-expression [79,77]. Immunoblotting with anti-KDEL (for GRP78) did not show an increase in the protein levels of this ER stress marker as a result of GFAT over-expression. The results suggest that an induction of ER stress response is not evident as there is likely not a sufficient level of glucosamine present to induce such a response – a similar observation to that seen in HepG2 cells.

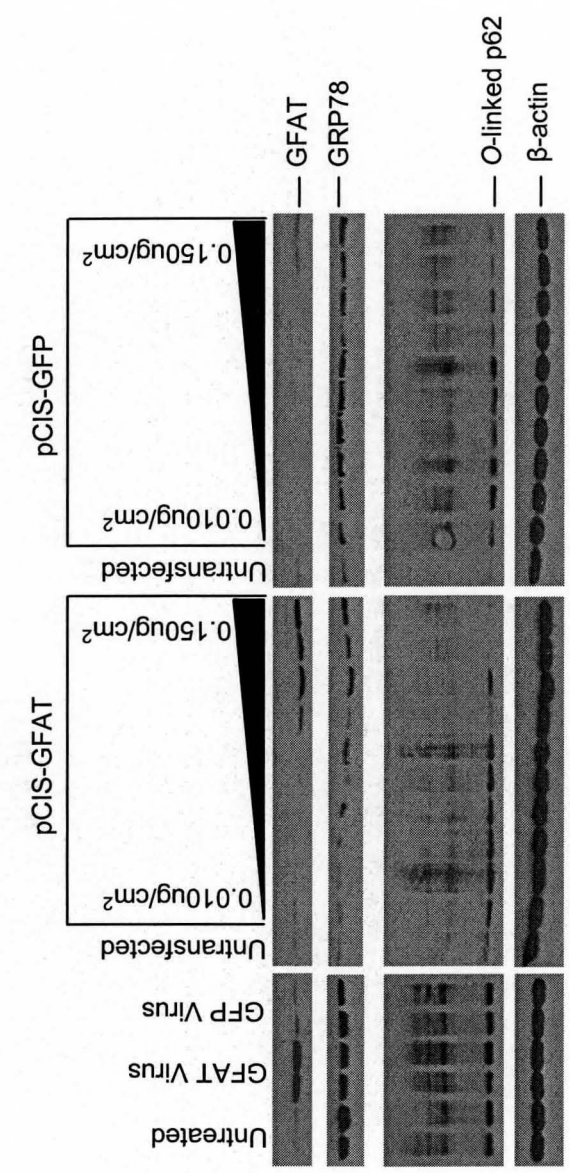


Figure 15.0 Western Blot Optimization and Analysis of GFAT Over-Expression in HASMC. **A)** A 100 moi was used to infect the HASMC with both GFAT/GFP and GFP adenoviruses. Cells were infected for 2 hours and allowed to grow in 231 media for 24 hours. HASMC were transiently co-transfected with **B)** pCIS-GFAT + pCIS-GFP and **C)** pCIS-GFP alone for 24 hours with the indicated amount of plasmid. GFAT expression was detected using GFAT primary antibody (a kind gift from Dr. C. Weigart, University of Tubingen, Germany). GRP 78 and HSP47 expression was detected using KDEL antibody. O-linked p62 was detected using RL2 antibody. β-actin loading control was detected using β-actin antibody.

Further optimization of the experimental conditions was used to assess of effect of GFAT over-expression on ER stress and protein glycosylation using immunofluorescence microscopy. Both pCIS-GFAT and the GFAT adenovirus increased the cellular levels of GFAT, but this was not sufficient to increase cellular glucosamine levels (indicated by protein *O*-linked glycosylation levels, and visualized with antibody 110.6) or induce an ER stress response. The results were similar to those seen by immunoblot (Data not shown).

3.2 The role of Protein Glycosylation in Glucosamine-induced ER Stress

3.2.1 Inhibition of *O*-GlcNAc Transferase: The effect of Alloxan on protein *O*-linked glycosylation and ER stress in HepG2 Cells.

Hyperglycemia and increased levels of glucosamine have been shown to increase the level of *O*-linked protein glycosylation and induce ER stress [59]. We theorized that the dysregulation of protein glycosylation may play a role in the induction of ER stress under hyperglycemic conditions. Given that increased cellular glucosamine levels increase protein *O*-linked glycosylation [79], we hypothesize that inhibiting the transfer of glucosamine to proteins may have an effect on ER stress induction. This effect is achieved through the use of an *O*-GlcNAc Transferase (OGT) inhibitor. Alloxan has been shown to be an effective agent in the inhibition of OGT and protein glycosylation [59,82,83,84]. In pancreatic beta cells and cultered fibroblasts, alloxan is a potent inhibitor of *O*-linked glycosylation when cells are treated with 2-5mM concentrations of the drug in the culture media [82,84]. Through the use of this compound, the effect of *O*-linked glycosylation inhibition on ER stress induction and was investigated in HepG2 cells (Figure 16.0).

Previously published findings report that a cell culture treatment with 2-5mM concentrations of alloxan is sufficient to inhibit the *O*-linked glycosylation of protein [59,83,84]. However, a 20mM concentration of alloxan was required in order to detect an effect with HepG2 cells. 20mM treatment of alloxan was unable to cause a complete block in *O*-linked glycosylation. This can be explained by the presence of some proteins

which are likely, constitutively *O*-linked glycosylated (and are therefore unaffected by changes in OGT activity). Immunoblot analysis of cell lysates indicates that glucosamine significantly increased *O*-linked glycosylation levels. For treatments with 20mM alloxan plus 5mM glucosamine, alloxan is able to prevent an increase in *O*-linked glycosylation and glycosylation levels were similar to that in controls and with alloxan alone (Figure 16.0 B). Additionally, it was found that 20mM alloxan was able to prevent a glucosamine-induced ER stress response, seen with both GRP78 and GADD 153 (Figure 16.0 C and D). The results suggest that Alloxan is an effective inhibitor of *O*-linked glycosylation in Hep G2 cells and that *O*-linked glycosylation may play a role in ER Stress induction.

ER stress inducing agents, tunicamycin and A23187 were used a positive ER stress controls. Neither agent had a significant effect on the *O*-linked glycosylation of proteins (Figure 16.0B). Both GRP78 and GADD153 expression were induced with tunicamycin and A23187 treatment (Figure 16.0 C and D). When cells were co-treated with alloxan and tunicamycin, there was a significant, but slight decreased in GRP78 expression – however, expression was still significantly greater than control (Figure 16.0 C). There was no change in GADD 153 expression as a result of alloxan and tunicamycin co-treatment. For the co-treatment of A23187 and alloxan, there was no effect on the either GRP78 or GADD 153 expression compared to A23187 alone (Figure 16.0 C and D).

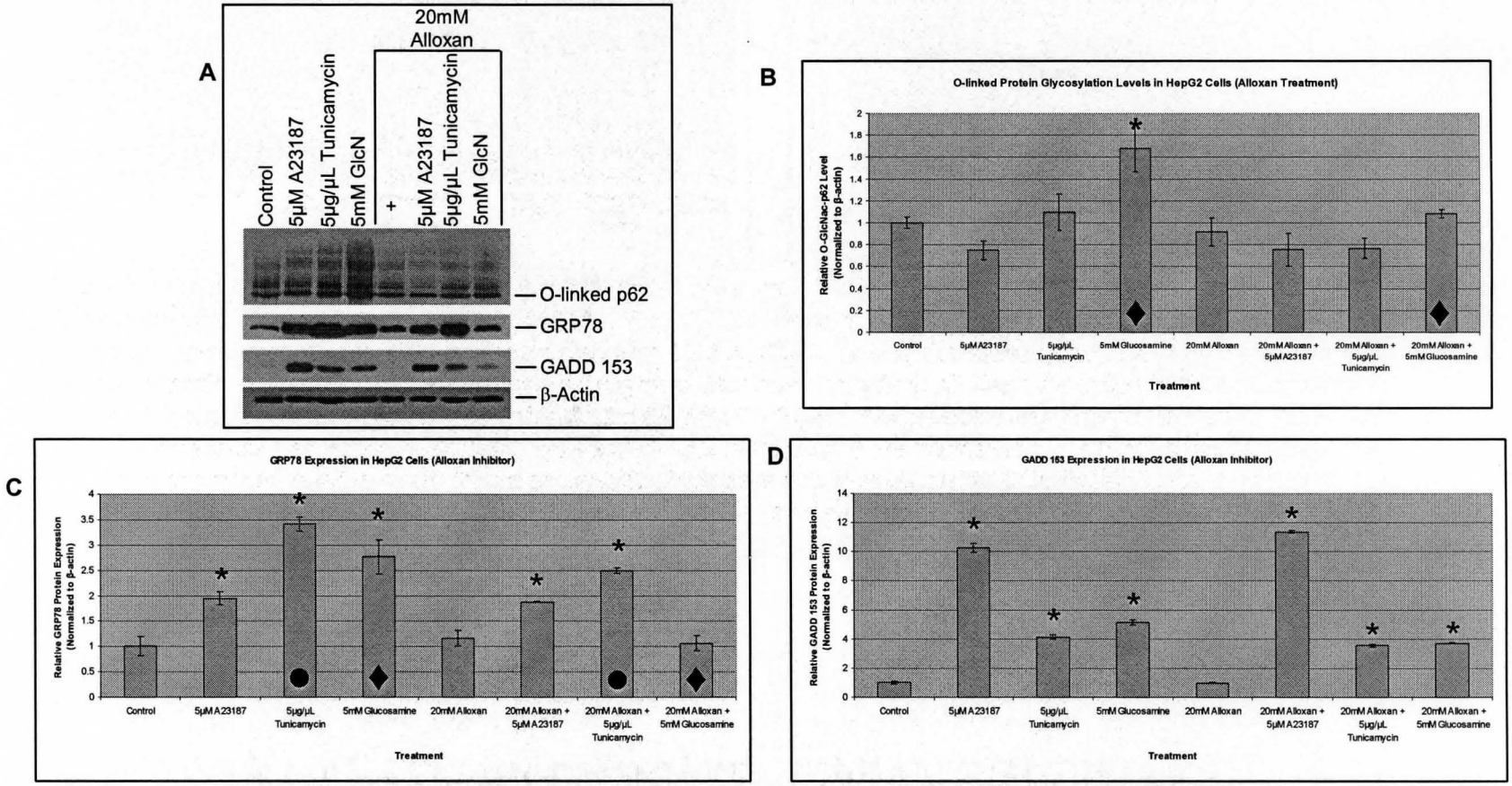


Figure 16.0 Alloxan Treatment of Hep G2 Cells. Hep G2 cells were treated for 24 hours with 20mM alloxan. Cells were then co-treated with with 20mM Alloxan and 5μM A23187, 5μg/μL tunicamycin and 5mM glucosamine (GlcN) for 24 hours. Cells which had not been treated with alloxan also treated with 5μM A23187, 5μg/μL tunicamycin and 5mM glucosamine for 24 hours as controls. Protein expression was determined by Western Blot and quantified. **A)** Representative blot from triplicate experiment. **B)** O-linked Glycosylation was determined using RL2 antibody. **C)** GRP 78 expression was determined using KDEL antibody. **D)** GADD 153 expression was determined using anti-GADD 153. All results were normalized to a β-actin loading control and fold change was determined relative to an untreated control. Error bars depict standard error in the triplicate experiment. * $p < 0.05$ compared to relative expression in respective control samples. ♦● samples which are significantly different from one another .

3.2.2 Inhibition of O-GlcNAcase: The effect of PUGNAc and "9c" on protein *O*-linked glycosylation and ER stress in HepG2 Cells.

The *O*-GlcNAcase inhibitors, PUGNAc and "9c" (see section 2.0 Materials and Methods) have been shown to be effective agents in the inhibition of protein deglycosylation – the removal of *O*-GlcNAc from proteins (Figure 3.0) [60]. This has the effect of increasing the level of cellular protein *O*-linked glycosylation [60]. This effect is similar to hyperglycemia and increased levels of glucosamine, which also increase the level of *O*-linked glycosylation [4,59]. To investigate further, whether increased *O*-linked glycosylation is responsible for inducing an ER stress response, we investigated the effect of PUGNAc and "9c" treatment on ER stress induction in HepG2 cells. Immunoblot analysis of cell lysates indicates that both PUGNAc and glucosamine can increase *O*-linked protein glycosylation to similar levels. ER stress inducing agents, tunicamycin and A23187 had no effect on *O*-linked glycosylation (Figure 17.0 B). PUGNAc treatment alone did not promote either GRP78 or GADD153 expression – diagnostic markers of ER stress, indicating that a general increase in *O*-linked glycosylation is not sufficient to induce ER stress. Treatment with free glucosamine, tunicamycin, and A23187, or in combination with PUGNAc did induce ER stress, indicating that PUGNAc treatment did not alter the ER stress response (Figure 17.0 C and D).

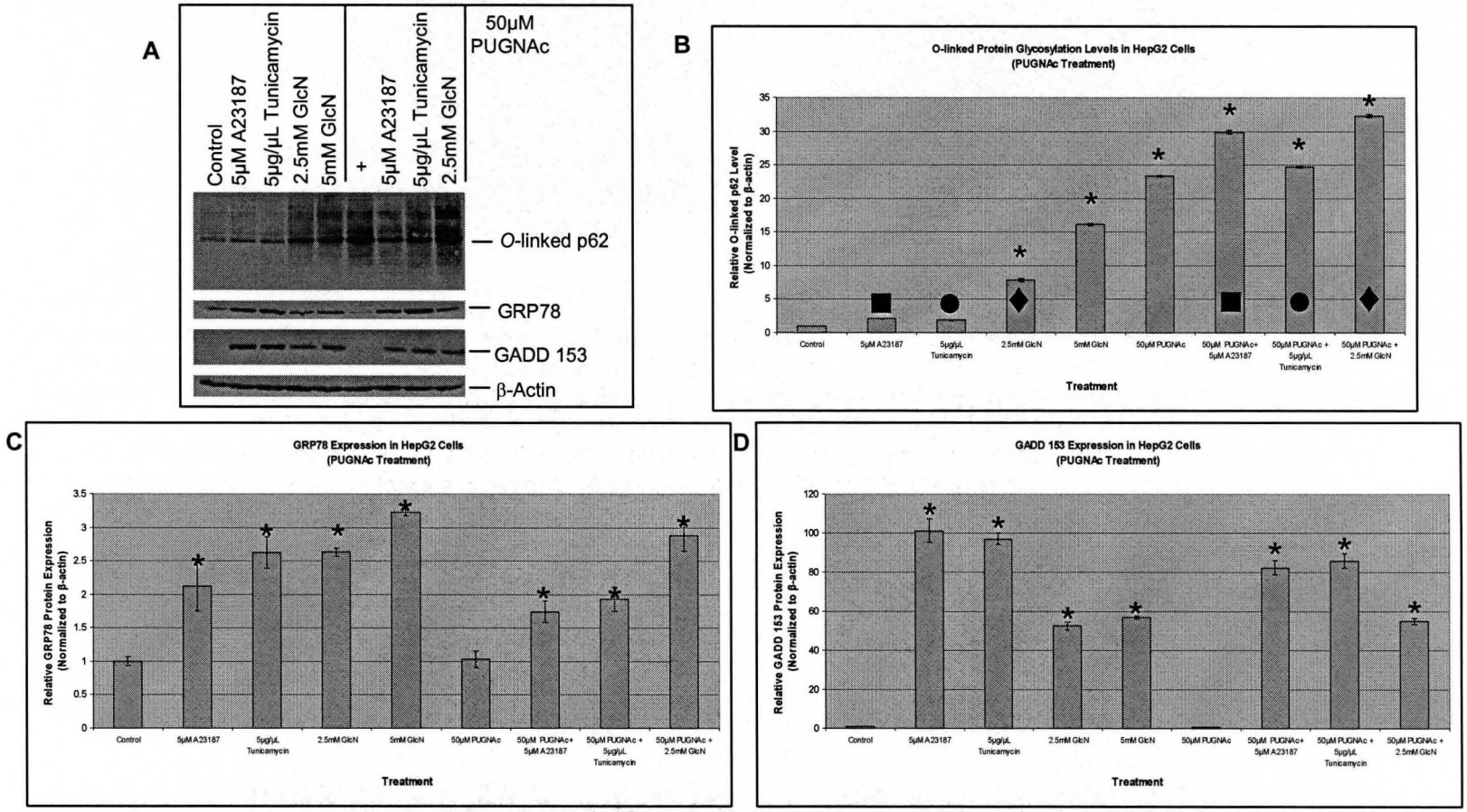


Figure 17.0 PUGNAc Inhibitor Treatment of Hep G2 Cells. Hep G2 cells were treated for 24 hours with 50μM PUGNAc. Cells were then treated with 5μM A23187, 5μg/μL tunicamycin and 2.5mM glucosamine for 8 hours. Cells which had not been treated with PUGNAc were also treated with 5μM A23187, 5μg/μL tunicamycin and 2.5 or 5mM glucosamine for 8 hours as controls. Protein expression was determined by Western Blot and quantified. **A)** Representative blot from triplicate experiment. **B)** *O*-linked glycosylation was determined using RL2 antibody. **C)** GRP 78 expression was determined using KDEL antibody. **D)** GADD 153 expression was determined using anti-GADD 153. All results were normalized to a β-actin loading control and fold change was determined relative to an untreated control. Error bars depict standard error in the triplicate experiment. * $p < 0.05$ compared to relative expression in respective control samples. ♦●■ samples which are significantly different from one another .

The results using "9c" inhibitor are similar to those with PUGNAc. Immunoblot analysis of cell lysates showed that both "9c" and glucosamine increased cellular levels of *O*-linked glycosylation and ER stress inducing agents, tunicamycin and A23187, had no effect on *O*-linked glycosylation (Figure 18.0 B). "9c" treatment did not promote either GRP78 or GADD153 expression. However, treatment of cells with free glucosamine, tunicamycin and A23187 alone or in combination with "9c" did induce ER stress (Figure 18.0 C and D). These results further support the experiments performed with PUGNAc and indicate that a general increase in protein *O*-linked glycosylation is not sufficient to induce an ER stress response.

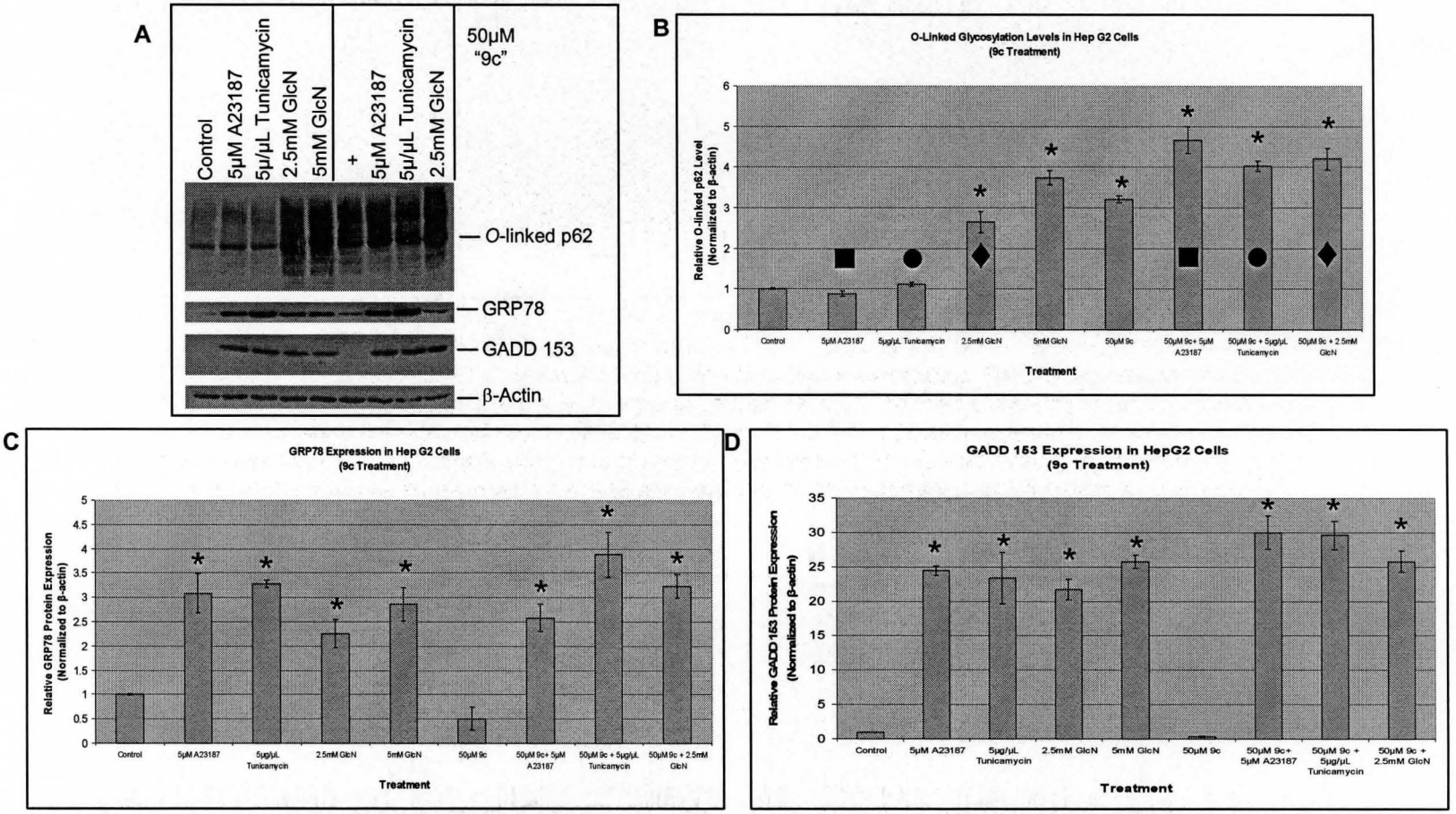


Figure 18.0 "9c" Inhibitor Treatment of Hep G2 Cells. Hep G2 cells were treated for 24 hours with 50μM 9c. Cells were then treated with 5μM A23187, 5μg/μL tunicamycin and 2.5mM glucosamine for 8 hours. Cells which had not been treated with 9c were also treated with 5μM A23187, 5μg/μL tunicamycin and 2.5 or 5mM glucosamine for 8 hours as controls. Protein expression was determined by Western Blot and quantified. **A)** Representative blot from triplicate experiment. **B)** *O*-linked glycosylation was determined using RL2 antibody. **C)** GRP 78 expression was determined using KDEL antibody. **D)** GADD 153 expression was determined using anti-GADD 153. All results were normalized to a β-actin loading control and fold change was determined relative to an untreated control. Error bars depict standard error in the triplicate experiment. * $p < 0.05$ compared to relative expression in respective control samples. ♦●■ samples which are significantly different from one another .

3.2.3 The effect of Glucosamine on protein *N*-linked glycosylation of eGFP-hSR-BI and α-1-AT in HepG2 Cells.

Glucosamine has been shown to affect the assembly of glycoprotein containing viruses [62]. We theorize that increase glucose flux through the hexosamine pathway may dysregulate polysaccharide production, and may have an effect on *N*-linked glycosylation. This would have drastic effects on folding and ER homeostasis – providing a possible explanation for the induction of ER stress and the UPR under hyperglycemic conditions. To investigate this hypothesis, the effect of glucosamine on the *N*-linked glycosylation of hSR-BI-eGFP and α-1-antitrypsin was investigated in HepG2 cells.

Immunoblot analysis of cell lysates indicates that glucosamine has an effect on the *N*-linked glycosylation of eGFP-hSR-BI. This effect is mimicked by a treatment of hSR-BI-eGFP with *N*-Glycosidase F and, to a similar extent, by Endoglycosidase H. Glucosamine treatment also induced an ER stress response – seen with an upregulation in GRP78 protein levels (Figure 19.0). These results suggest that increased levels of cellular glucosamine affect protein *N*-linked glycosylation and may induce ER stress through a mechanism involving dysregulated protein folding and processing in the ER.

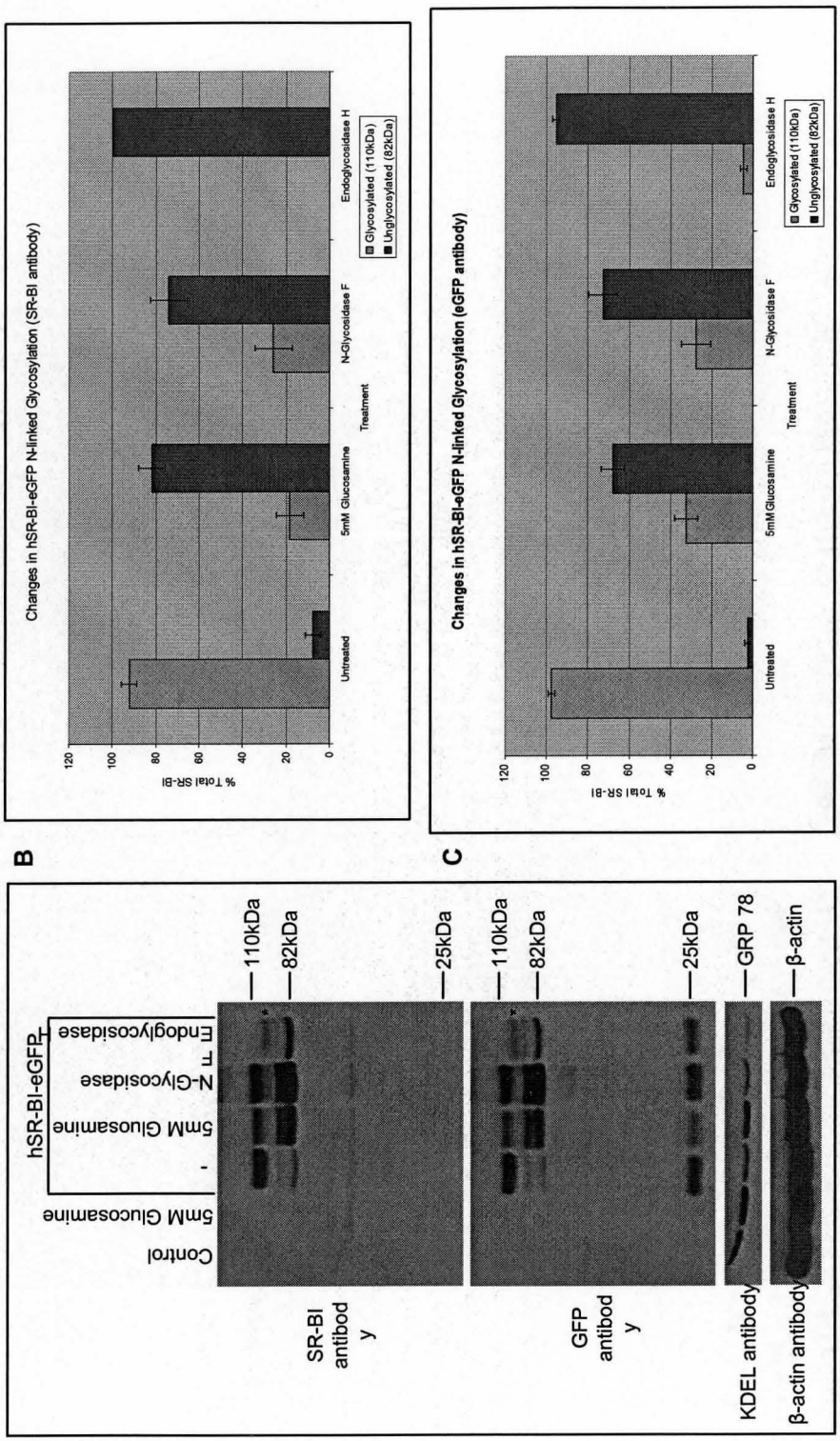


Figure 19.0 Western Blot Analysis of hSR-BI-eGFP *N*-linked Glycosylation in Hep G2 Cells. Hep G2 cells were transiently transfected with pSR-BI-eGFP for 24 hours. Cells were then treated with 5mM glucosamine for a subsequent 24 hours. Lystates of eGFP-hSR-B transfected cells were then treated with N-Glycosidase F or endoglycosidase H for 16 hrs at 37°C. **A)** a representative blot from a triplicate experiment is shown. hSR-BI-expression was detected using SR-BI antibody and eGFP antibody. GRP 78 expression was determined using KDEL antibody. β-actin loading control was detected using β-actin antibody. *Indicates mature, Endo H resistant hSR-BI-eGFP. Changes in hSR-BI-eGFP protein glycosylation were determined as a percentage of total hSR-BI-eGFP expression using both **B)** SR-BI and **C)** eGFP antibodies. Levels of glycosylated vs unglycosylated are significantly different from Untreated control. ($p < 0.05$ compared with relative expression in control samples) Error bars depict standard error in the triplicate experiment.

Immunoblot analysis of cell lysates was also used to study α-1-AT. Glucosamine had no effect on the *N*-linked glycosylation of α-1-AT. However, glucosamine treatment did increase cellular *O*-linked glycosylation levels and GRP78 protein levels (Figure 20.0). Treatment of α-1-AT with *N*-Glycosidase F yields three bands – indicative of the multiple sites of *N*-linked glycosylation present on α-1-AT. α-1-AT is known to have 3 sites of *N*-linked glycosylation [71]. Treatment of α-1-AT with Endoglycosidase H had no effect on the *N*-linked sugars – indicating that α-1-AT present in the lysates were fully mature proteins, containing only complex, Endo H-resistant glycans.

Figure 20.0 Western Blot Analysis of α-1-antitrypsin *N*-linked Glycosylation inHepG2 Cells.

Lysates from Hep G2 cells transiently transfected with pSR-BI-eGFP were also used to investigate α-1-antitrypsin. Cells were treated with 5mM glucosamine for 24 hours and lystates were then treated with N-Glycosidase F or endoglycosidase H for 16 hrs at 37°C.

A) A representative blot from a triplicate experiment is shown. α-1-antitrypsin expression was detected using α-1-antitrypsin antibody. GRP78 expression was determined using KDEL antibody. *O*-linked glycosylation was determined using RL2 antibody. **B)** Changes in α-1-antitrypsin protein glycosylation were determined as a percentage of total α-1-antitrypsin using α-1-antitrypsin antibody. Results were normalized to a β-actin control. Levels of glycosylated vs. unglycosylated 1& 2 in N-Glycosidase F treated samples are significantly different from controls. Level of glycosylated α-1-antitrypsin in glucosamine treated group is significantly different from controls. (* $p < 0.05$ compared with relative expression in control samples). All other samples are not significantly different from respective controls. Error bars depict standard error in the triplicate experiment.

4.0 Discussion

4.1 The role of GFAT in Hyperglycemia-associated ER Stress

There is an abundance of clinical data outlining the relationship between diabetes mellitus, atherosclerosis and its clinical complications – what continues to elude the research community, is how hyperglycemia perpetuates vascular disease [85,86]. Studies in both cell culture and animal models of diabetes have implicated the hexosamine pathway in the pathogensis of the downstream complications hyperglycemia [37]. Multiple enzymes facilitate the conversation of glucose to glucosamine and its UDP-conjugated metabolites (Figure 1.0) and only glutamine:fructose-6-phosphate amidotransferase (GFAT) is considered to be rate-limiting in this process [3].

Additionally, it is known that glucosamine contributes to cellular dysfunction by interfering with ER homeostasis and inducing the UPR [39]. We hypothesized that the hexosamine pathway and GFAT may play a role in stimulating ER stress, particularly under hyperglycemic conditions [2,39].

The liver is a primary site of glucose metabolism in the body. It facilitates glycogen storage and glucose release, during fasting periods between meals. During periods of starvation, it is the site of gluconeogenesis, and is required to meet the glucose demands of the brain. But most significantly, hepatic tissue is sensitive to glucose. In this study, we have examined the hexosamine pathway in multiple cells types. Initial experiments were performed in HepG2 cells. HepG2 cells are a hepatocarcinoma cell

line and therefore differ from primary hepatocytes, however, this cell line retains many hepatic characteristics and therefore has relevance in the study of hyperglycemia and the hexosamine pathway.

We first set out to examine the effects of the simple sugars and amino acids required in the hexosamine pathway, with regard to their potential ER stress inducing effect. Our studies in HepG2 cells indicated that glucosamine had an ER stress-inducing effect. Contrary to our previous findings, elevated levels of glucose did not induce ER stress [4]. Furthermore, this cell line appeared to be able to regulate its production of glucosamine from glucose. There is evidence from *in vitro* studies showing that glucosamine-6-phosphate and UDP-GlcNAc are able to inhibit GFAT [34]. These results suggest that feedback inhibition of GFAT may occur under hyperglycemic conditions, as a method of shunting glucose into glycolysis and glycogen synthesis.

We investigated whether GFAT over-expression would be sufficient to overcome any possible enzymatic regulation and increase cellular glucosamine levels, inducing an ER stress response. We examined the effect of transient GFAT over-expression in HepG2 cells on ER stress response and protein *O*-linked glycosylation. In all experiments, studying both mRNA and protein expression, we found that GFAT over-expression was not sufficient to induce expression of GRP78, an ER stress marker, nor was it sufficient to affect the cellular level of protein *O*-linked glycosylation. Theorically, hyperglycemic conditions would act in concert with GFAT over-expression to exacerbate an effect of increased glucosamine production. Interestingly, no effect was observed when GFAT transfected cells were supplied with 30mM glucose. *O*-linked

glycosylation levels were increased as a result of the hyperglycemic conditions but this was comparable to control. The lack of an increase in protein *O*-linked glycosylation suggests that GFAT over-expression does not significantly increase cellular levels of glucosamine in HepG2 cells. This result provides a possible explanation for the lack of induced ER stress response observed with GFAT over-expression. To further these studies, we decided to investigate additional cell types in order to identify cells that can initiate an detectable ER stress response and have an active hexosamine pathway – that is sensitive to both glucose and glucosamine. HASMC and HEK 293 cells were chosen for examination.

HASMC are particularly relevant to the study of atherosclerosis. HEK293 cells were chosen as an alternate cell type, that is particularly useful for using viral methods of protein over-expression, and may be good for studying GFAT over-expression using the adenovirus obtained from Dr. H. Kaneto, Joslin Diabetes Centre, Boston, MA, USA. Both cell lines were examined with regards to their glucose and glucosamine sensitivity, in order to determine their suitability for further GFAT over-expression studies. The ER stress reponse and protein *O*-linked glycosylation of HASMC were found to be mildly sensitive to both glucose and glucosamine treatment. HEK 293 cells were found to be generally insensitive to either glucose or glucosamine. As a result, HASMC were used for further GFAT over-expression experiments.

The conditions were optimized and GFAT was successfully over-expressed, using both transient transfection of a CMV driven GFAT and also using an adenoviral construct. Both the pCIS-GFAT construct and the GFAT adenovirus significantly

increased GFAT mRNA and protein expression levels. However, over-expression had no effect on the expression of the diagnostic marker of ER stress, GRP78. In addition, the cellular level of protein *O*-linked glycosylation was not significantly affected. These results that are similar to observations made in HepG2 cells.

The results collected in both HepG2 cells and HASMC neither support nor discount our hypothesis that GFAT and the hexosamine pathway may play a role in hyperglycemia-associated ER stress. We have shown that it is possible to over-express GFAT in these cell types, however, the GFAT appears non-functional, given that we don't see changes in the cellular levels of *O*-linked glycosylation. These results were not expected. The GFAT over-expression systems we have used, have also been utilized in other cell types – to study other factors associated with diabetes – with great success [76,78,87,88]. However, it also worth noting that our methods, though widely accepted, do not directly assess GFAT activity – and this will be the aim of future studies investigating GFAT over-expression in these and other cell lines [4,79,89].

4.2 The role of Protein Glycosylation in Glucosamine-induced ER Stress

Glucosamine is a well-established ER stress-inducing agent, which also plays an essential role in both *N*-linked and *O*-linked protein glycosylation [39,59,61]. Our results, as well as others, show that hyperglycemic conditions and glucosamine, increase cellular *O*-linked glycosylation levels in multiple cell types [59]. This can have a number of intracellular effects, given the dynamic role of *O*-linked glycosylation in multiple cellular processes. There is also evidence that glucosamine has an effect on protein *N*-linked glycosylation [39,62,64]. *N*-linked glycosylation facilitates a number of processes intracellularly, but its involvement in the folding of newly synthesized proteins makes it an important event for maintaining ER homeostasis [61]. We, therefore, hypothesizd that protein glycosylation (*O*-linked, *N*-linked, or both) may play a role in glucosamine-induced ER stress and cellular dysfunction.

Experiments investigating the role of protein *O*-linked glycosylation in glucosamine-induced ER stress have yielded some interesting findings. Studies utilizing alloxan as an inhibitor of protein *O*-linked glycosylation have led to multiple observations. First, the inhibitor did not completely block *O*-linked glycosylation of proteins under euglycemic conditions. This suggests that there is a likely a constitutive cellular level of protein *O*-linked glycosylation. Alloxan did, however, prevent a glucosamine-induced increase in protein *O*-linked glycosylation. This coincided with a diminished, glucosamine-induced expression of GRP78 and GADD 153. The results

suggest that by inhibiting protein *O*-linked glycosylation, we are able to protect cells from glucosamine-induced ER stress.

We also studied the effect of the *O*-GlcNAcase inhibitors, PUGNAc and "9c", on ER stress induction and protein *O*-linked glycosylation. Here we found that elevated levels of *O*-linked glycosylation do not correspond to elevated levels of ER stress markers. These findings suggest that *O*-linked glycosylation is not sufficient to directly promote ER stress. However, PUGNAc and "9c" cause a general increase in the level of *O*-linked glycosylation of proteins – proteins which would be glycosylated under normal cellular conditions. It is possible that increased cellular glucosamine levels cause the *O*-linked glycosylation of *specific* proteins, which initiate or signal an ER stress response. If this were the case, a general increased in the basal level of glycosylation may not have an effect on ER stress induction and the inhibition of glycosylation, would prevent a glucosamine-induced ER stress response. Neither alloxan, PUGNAc or "9c" had a profound effect on ER stress induced with either tunicamycin or A23187, further supporting this observation.

While we have made some interesting observations, we cannot make any substantial conclusions as to how *O*-linked glycosylation mediates ER stress-induction. We can hypothesize that there are specific, *O*-link glycosylated protein intermediates, however our results do not shed light on the identity of these possible mediators. It has been shown that hyperglycemic conditions increases the *O*-linked glycosylation of a number of proteins. As previously described, hyperglycemic conditions increase the glycosylation of the transcription factor, Sp1, inhibiting its activity and degradation by

the 26S proteasome [53,57,58]. Glycosylation of the proteasome itself has also been shown to decrease its protein degradation capabilities [58]. It is possible that the glucosamine-induced *O*-linked glycosylation of other proteins may lead to the dysregulation of their degradation as well, thus, possibly contributing to an accumulation of unfolded protein in the ER and activating the UPR. However, this hypothesis requires further investigation. Proteomic techniques may be very useful in this line of investigation for future studies.

In addition to studying *O*-linked glycosylation, we also investigated the effect of glucosamine on *N*-linked protein glycosylation. *N*-linked glycosylation is required for protein folding in the ER [61]. Dysregulation of this event would be a possible explanation for the induction of ER stress and the UPR under hyperglycemic conditions. Qui *et al.* has previously shown that glucosamine treatment of HepG2 cells, inhibits the *N*-linked glycosylation of ApoB-100 and α_1-AT [63]. Vivienne Tedesco has also previously shown that glucosamine treatment of RAW 264.7 and ldlA-7 cells showed that the *N*-linked glycosylation of mouse SR-BI is also prevented [64]. To further these studies, we investigated the effect of increased cellular glucosamine levels on the *N*-linked glycosylation of human Scavenger Receptor – BI (hSR-BI) and α_1-AT in HepG2 cells, by immunoblot.

Our studies of eGFP- hSR-BI indicate that glucosamine causes a dysregulation of protein *N*-linked glycosylation of SR-BI. Glucosamine caused a shift in the molecular weight of the protein, towards that of the unglycosylated form. This effect on SR-BI was

mimicked by treatment with *N*-Glycanase F and Endoglycosidase H, which remove *N*-linked glycans.

Our studies of α-1-AT showed that glucosamine did not have an effect on its *N*-linked glycosylation. These results contrast those with SR-BI and those of Qiu *et al.*, who observed that glucosamine prevents the glycosylation and the processing of the *N*-linked glycans of α-1-AT [74]. However, Qiu *et al.* used a pulse-chase method to study newly synthesized α-1-AT [74]. We do know that the α-1-AT in our experiments was insensitive to Endoglycosidase H treatment, indicating the protein has fully mature, complex oligosaccharides. In contrast, not all of the SR-BI in our experiments was insensitive to Endoglycosidase H treatment, indicating that we are studying both mature and newly synthesized SR-BI. By comparison, our results suggest that increased levels of cellular glucosamine do not affect fully processed *N*-linked glycans but likely newly glycosylated proteins – likely those which are newly synthesized and associated with the ER. Further experiments are required to test this hypothesis. Experiments studying the effect of glucosamine on both newly synthesized, and fully mature proteins – for both hSR-BI-eGFP, α-1-AT as well as other potential glycoprotein targets – need to be investigated.

5.0 Conclusion

Diabetes mellitus and CVD will continue to be major factors contributing to morbidity and mortality in the developed world in the decades to come. Although many theories and molecular mechanism have emerged, linking diabetes mellitus to CVD, this field continues to expand and we continue to search for novel and effective interventions in the treatment of these diseases. We have now identified the hexosamine pathway and protein glycosylation as cellular players in ER stress induction. From these investigations, we now know that glucose flux through the hexosamine pathway is a highly regulated process – one that still requires intense investigation. Additionally, we have identified that both *O*-linked and *N*-linked glycosylation are affected by increased levels of cellular glucosamine and may play a contributing role in the induction of ER stress under hyperglycemic conditions. Further studies are still required and are essential to expanding our understanding of the roles of the hexosamine pathway and protein glycosylation in ER stress induction. However, given the established link between hyperglycemia, ER stress and atherosclerosis, the continued study and characterization of these pathways will only further our knowledge base and have the potential to aid in the development of effective diabetes and CVD therapies in the future.

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