The role of the hexosamine biosynthesis pathway in control of hepatic glucose metabolism

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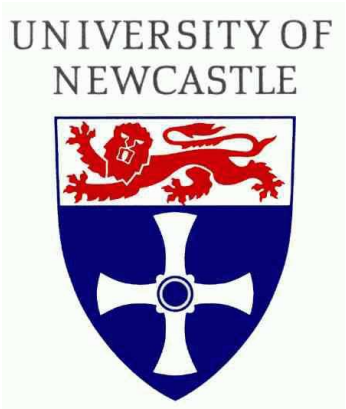

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Abstract

Type 2 diabetes is associated with increased hepatic lipogenesis and glucose production. Enzymes of lipogenesis are co-ordinately induced by insulin and glucose. However, the enzyme glucose 6-phosphatase (G6Pc), which catalyses the final reaction in hepatic glucose production is repressed by insulin but induced by glucose and is markedly elevated in type 2 diabetes. Another gene that is repressed by insulin and induced by glucose in muscle is thioredoxin interacting protein (TXNIP), which is abnormally elevated in muscle in type 2 diabetes. TXNIP gene regulation in liver has not been reported. The induction of hepatic lipogenic enzymes by glucose is attributed to the transcription factor ChREBP-Mlx, whereas the glucose-induction of G6Pc is attributed to covalent modification of FOXO transcription factors by O-GlcNAc formed by the hexosamine biosynthesis pathway (HBP). The aim of this thesis was to investigate the role of the HBP in regulation by glucose of G6Pc and TXNIP gene expression in hepatocytes.

This thesis investigated three commonly used methods to modulate HBP flux and covalent modification by O-GlcNAc. (1) An inhibitor of glutamine:fructose 6-phosphate amidotransferase (6-diazo-5-oxo-l-norleucine, DON), the rate limiting enzyme of the HBP, was established to be a valid tool to study glucose-regulated gene expression. (2) Substrates that enter the HBP after GFAT, such as glucosamine which is widely used to demonstrate links between HBP or O-GlcNAc modification and insulin resistance were shown to be invalid tools. (3) Inhibitors of O-GlcNAc modification or expression of O-GlcNAc transferase were of limited use to alter protein modification by O-GlcNAc.

Glucose caused a larger induction of G6Pc and TXNIP mRNA in the absence of insulin than in its presence, and this induction could be largely accounted for by Mlx-dependent mechanisms (supporting involvement of ChREBP or MondoA) and by FOXO transcription factors. G6Pc and TXNIP expression were confirmed to be regulated by distinct mechanisms based on the induction of TXNIP but not G6Pc by the glucose analogue, 2-deoxyglucose, through an Mlx-independent mechanism. Insulin caused rapid translocation of both FOXO1 and FOXO3A from the nucleus to the cytoplasm. Both glucose and 2-deoxyglucose opposed the translocation of FOXO1 and FOXO3A by insulin, and they stimulated translocation of FOXO3A to the nucleus in the absence of insulin.

Inhibition of HBP flux with the GFAT inhibitor had the following effects: (i) counteraction of the glucose-induction of both G6Pc and TXNIP mRNA; (ii) counteraction of glucose-induced translocation of ChREBP to the nucleus without affecting the signalling metabolite, fructose 2,6-bisphosphate; (iii) counteraction of glucose-induced translocation of FOXO1 and FOXO3A to the nucleus. A role for O-GlcNAc modification of both ChREBP and FOXO3A was supported by wheat-germ agglutinin precipitation. The results of this thesis support involvement of both HBP flux and O-GlcNAc modification of ChREBP and FOXO3A in glucose-regulated expression of G6Pc and FOXO3A but they do not support a role for glucosamine as an experimental tool to study glucose-induced insulin resistance.

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Contents

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This thesis investigated three commonly used methods to modulate HBP flux and covalent modification by O-GlcNAc. (1) An inhibitor of glutamine:fructose 6-phosphate amidotransferase (6-diazo-5-oxo-l-norleucine, DON), the rate limiting enzyme of the HBP, was established to be a valid tool to study glucose-regulated gene expression. (2) Substrates that enter the HBP after GFAT, such as glucosamine which is widely used to demonstrate links between HBP or O-GlcNAc modification and insulin resistance were shown to be invalid tools. (3) Inhibitors of O-GlcNAc modification or expression of O-GlcNAc transferase were of limited use to alter protein modification by O-GlcNAc.

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Abbreviations

2-DOG	2-deoxyglucose
3-MOG	3-O-methylglucose
5TG	5-Thioglucose
6-DOG	6-deoxyglucose
ACC	Acetyl Coenzyme A carboxylase
AD	Actinomycin D
Acetyl- CoA	Acetyl Coenzyme A
Acetyl-H4	Acetyl-Histone 4
AMP	Adenosine 5'-monophosphate
AMPK	Adenosine 5'-monophosphate-activated protein kinase
ATP	Adenosine 5'-triphosphate
AZN	Azaserine
BADGP	Benzyl-2-acetamido-2-deoxy-alpha-D-galactopyranoside
BSA	Bovine serum albumin
cAMP	Adenosine 3', 5'-cyclic monophosphate
ChIP	Chromatin imunoprecipitation
ChoRE	Carbohydrate response element
ChREBP	Carbohydrate response element binding protein
CREBP	cyclic AMP response element binding protein
CRTC2	CREB regulated transcription coactivator 2
CX	cycloheximide
DAPI	4',6-diamidino-2-phenylindole
db-cAMP	Dibutyryl adenosine 3', 5'-cyclic monophosphate
DEC1	Transcriptional repressor encoded by BHLHB2
DEC2	Transcriptional repressor encoded by BHLHB3
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-Deoxynucleotide 5'-triphosphate
DTT	DL-Dithiothreitol
DON	6-Diazo-5-oxo-L-norleucine
EDTA	Ethylenediaminetetraaceticacid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic Acid
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FOXO1	Forkhead box protein O1
FOXO3A	Forkhead box protein A3
Fructose 1-P	Fructose 1-phosphate
F 2,6-P_2	Fructose 2,6-bisphosphate
Fructose 6-P	Fructose 6-phosphate
G3PDH	Glycerophosphate dehydrogenase
G6PT	Glucose 6-phosphate transporter
G6Pc	Glucose 6-phosphatase

G6PDH	Glucose 6-phosphate dehydrogenase
GalN	Galactosamine
Gck	Glucokinase
GFAT	Glutamine: fructose-6-phosphate amidotransferase
GFM	Glucose-free media
GFP	Green fluorescent protein
GK	Glucokinase
GKA	Glucokinase activator
GKRP	Glucokinase regulatory protein
Glc	Glucose
GlcN	Glucosamine
GlcN 6-P	Glucosamine 6-phosphate
GlcN 6-P NATase	Glucosamine 6-phosphate N-acetyltransferase
Gln	Glutamine
Glucose 1-P	Glucose 1-phosphate
Glucose 6-P	Glucose 6-phosphate
GLUT	Glucose transporter
GLUT2	Glucose transporter 2
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase-3
GP	Glycogen phosphorylase
GP-a	Phosphorylated active form of glycogen phosphorylase-(a)
GP-b	Dephosphorylated inactive form of glycogen phosphorylase-(b)
HBP	Hexosamine biosynthesis pathway
HDAC	Histone deacetylase
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2'ethanesulphonic acid)
Hexose 6-P	Hexose 6-phosphate
HIF1α	Hypoxia-inducible factor alpha
HNF4-α	Hepatocyte nuclear factor 4 alpha
IF	immunofluorescence
IGFBP-1	Insulin-like-growth-factor-binding protein 1
IR	Insulin receptor
IRs	Insulin receptor substrate
kDa	Kilodalton
LXR	Liver X rceptor
Mad	Transcriptional repressor Mad
Mad4	Transcriptional repressor Mad4
Malonyl-CoA	Malonyl Coenzyme A
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MethN	Methylamine
Mlx	Max-like protein X
Mlx-DN	Dominant negative variant of max-like protein X
Mnt	Max binding protein
MondoA	Mlx-interacting protein
mRNA	Messenger ribonucleic acid
NAD	β-Nicotinamide adenine dinucleotide
NADH	β-Nicotinamide adenine dinucleotide, reduced
NADP	β-Nicotinamide adenine dinucleotide phosphate
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced

NAG	N-acetylglucosamine
NAG 6-P	N-acetylglucosamine 6-phosphate
NF-Y	Nuclear factor Y
OGA	β-N-acetylglucosaminidase
O-GlcNAc	O-linked N-acetylglucosamine
O-GlcNAcylation	O-linked N-acetylglucosamine modification
OGT	O-linked N-acetylglucosamine transferase
p300	Histone acetyltransferase p300
PBS	Phosphate buffered saline
PCA	Perchloric acid
PCR	Polymerase chain reaction
PD169316	4-(4-Fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole
PFK	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1
Pi	Inorganic phosphate
PMSF	Phenylmethylsulfonyl fluoride
PPi	Pyrophosphate
PEPCK/Pck1	Phosphoenolpyruvate carboxykinase 1
L-PK/Pklr	Pyruvate kinase, liver and red blood cells
PFK-KD	Kinase deficient variant of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1
PFK-WT	Wild type form of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1 (liver isoform)
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PhK	Phosphorylase kinase
PKA	Protein kinase A
PKB/Akt	Protein kinase B
PP1	Protein phosphatase-1
PPi-PFK	Pyrophosphate: fructose 6-phosphate phosphotransferase
Ptdlns(3, 4, 5)P3	Phosphatidylinositol 3,4,5-trisphosphate
PTG	Protein targeting to glycogen
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
S4048	(1-[2-(4-Chloro-phenyl)-cyclopropylmethoxy]-3, 4-dihydroxy5-(3-imidazo[4, 5-b]pyridin-1-yl-3-phenyl-acryloyloxy)cyclohexanecarboxylic acid)
SDS	Sodium dodecyl sulphate
sh-RNA	Short hairpin ribonucleic acid
SREBP-1c	Sterol regulatory element-binding protein-1c
TBP2	thioredoxin binding protein 2
TCA	Trichloroacetic acid
Temed	Tetramethylethylenediamine
TGF-β	The transforming growth factor beta
TM	Tunicamycin
TPI	triosephosphate isomerase
Tris	Tris(hydroxymethyl)amino-methane
TSA	Trichostatin A

TRX	Thioredoxin
TXNIP	Thioredoxin interacting protein
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
UDP-glucose	Uridine diphosphoglucose
WB	Western Blotting
WGA	Wheat germ agglutinin
Xylulose 5-P	Xylulose 5-phosphate

Chapter 1

Introduction

Introduction

1.1 General introduction on diabetes

Diabetes mellitus is a heterogeneous group of disorders characterized by high blood glucose concentrations and associated abnormalities of lipid and protein metabolism. Insulin function is abnormal in diabetes, because of reduced secretion and tissue insensitivity to its effects. There are two main types, type 1 (insulin-dependent) and type 2 (non-insulin-dependent), although an individual can be assigned to only one class, that designation may change with time (Holt, 2004). The current classification is hybrid, based largely on clinical features at diagnosis (e.g. type 1 and 2) but also on aetiological mechanisms or associations [e.g. malnutrition-related (MRDM) or gestational (GDM) classes]. While there are broad relationships between pathogenic mechanisms and certain types of diabetes, the connections are far from absolute. For example, autoimmune damage inflicted on the pancreatic (beta-cells) is usually responsible for type 1, but may be associated with type 2, impaired glucose tolerance (IGT) or indeed with normal glucose tolerance (Harris, 1988).

The outcome of patients suffering from diabetes mellitus dramatically changed with the introduction of insulin therapy. However, with time it became clear that diabetic patients treated with insulin developed a series of complications that significantly reduced the quality and extent of life. The tissues and organ systems that were found to be affected are diverse including the retina, kidney, macro- and micro-vascular, and peripheral nerves (Ahmed, 2005). There are many theories explaining the development of these complications and take into account the

potential role of hyperglycemia, the lack of insulin and an imbalance of other hormones (Brownlee, 2001).

One theory proposes a link between the hexosamine biosynthesis pathway (HBP) and insulin resistance (Buse, 2006). The aim of this thesis was test the hypothesis that the HBP in the liver has a role in glucose sensing and the regulation of key enzymes of glucose metabolism, glucose 6-phosphatase and TXNIP through regulation of expression of these genes.

1.2 The role of the liver in the control of glucose metabolism

The liver plays an important role in controlling the blood glucose concentration and this process is regulated by the pancreatic hormones insulin and glucagon. After a meal, increasing concentrations of glucose in plasma stimulate the beta-cells in the pancreas to produce insulin and this stimulates glucose metabolism by liver, muscle and adipose tissue. High glucose uptake favours the pathways of glucose storage as glycogen synthesis, and also glycolysis to produce pyruvate which in liver and adipose tissue is then converted to fatty acid and triglyceride, glucose is also metabolised by the pentose mono-phosphate shunt to generate NADPH for lipid synthesis (Hers, 1976; Ferrer et al., 2003; Postic et al., 2004). Insulin stimulates conversion of glucose to glycogen and also to triglyceride both by rapid mechanisms involving covalent modification of proteins and by regulation of gene expression. On the other hand, alpha cells in the pancreas produce glucagon when glucose concentrations decrease during fasting and this stimulates glucose production by glycogenolysis and gluconeogenesis through changes in covalent modification of proteins and control of gene expression (Hers, 1976).

1.2.1 Regulation of glucose production and glycogen synthesis

During fasting when glucose is not being absorbed from the gut / intestine, the liver maintains the blood glucose concentration by release of glucose by two pathways, glycogenolysis and gluconeogenesis pathways. The liver produces glucose from glycogen by glycogenolysis which involves phosphorolysis to form glucose 1-phosphate and also from gluconeogenesis pathway from non-carbohydrate substrates such as lactate, amino acids and glycerol to glucose. This pathway is very important when the liver glycogen stores have been depleted. The activities of unidirectional enzymes of gluconeogenesis, for example, phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose 6-phosphatase (G6Pc) are regulated at the transcriptional level by insulin and glucagon (Postic et al., 2004).

Several enzymes and proteins play an important role in the regulation of hepatic glycogen synthesis from glucose. Transport of glucose into the liver cells is by a glucose transport protein (GLUT2) which has a high activity and maintains equilibrium of glucose between intracellular and extracellular sinusoidal compartment. The first reaction of glucose metabolism is phosphorylation to glucose 6-phosphate (glucose 6-P), which is catalysed by glucokinase (GK) (Agius et al., 2008). Glucose 6-P is a substrate for glycolysis, glycogen synthesis and pentose phosphate pathway (Ferrer et al., 2003; Postic et al., 2004). Fructose 6-phosphate which is produced from glucose 6-phosphate by phosphoglucose isomerase (PGI) is a substrate for glycolysis and also for the hexosamine biosynthesis pathway.

Glycogen metabolism is controlled by key regulatory enzymes. Glycogen degradation is dependent on active glycogen phosphorylase (GP). Glycogen phosphorylase occurs in two forms: an active phosphorylated form (phosphorylase-a) and an inactive dephosphorylated form (phosphorylase-b). Phosphorylase kinase converts glycogen phosphorylase-b to phosphorylase-a, by phosphorylation of a serine residue (Ser-14) at the N-terminus. Phosphorylase kinase is a hexadecamer composed of four sub-units. The alpha and beta-subunits have an inihibitory effect on the catalytic unit. They are regulated by phosphorylation by cAMP-dependent protein kinase, which releases the inhibition causing activation of the catalytic unit (Bollen et al., 1998). cAMP-dependent protein kinase is a downstream target of glucagon signalling in liver cells. In contrast, insulin signalling stimulates the activity of phosphorylase phosphatase which is composed of protein phosphatse-1 (PP-1) in association with glycogen targeting subunits (including protein targeting to glycogen (PTG)). Activation of phosphorylase phosphatase causes inactivation of glycogen phosphorylase-a by dephosphorlyation to phosphorylase-b. Glycogen synthesis is regulated by changes in covalent modification of both glycogen phosphorylase and glycogen synthase. Glycogen synthesis is controlled by reversible phosphorylation on multiple sites. Phosphorylation by multiple kinases including cAMP-dependent protein kinase, phosphorylase kinase, glycogen synthase kinase-3 and AMP-activated protein kinase causes inactivation by phosphorylation to form glycogen synthase-b (GSb). Glycogen synthase is activated by dephosphorylation by glycogen synthase phosphatase (GSP) which comprises protein phosphatase-1 in association with glycogen targeting proteins (including GL). Dephosphorylation causes activation to glycogen-synthase-a (Bollen et al., 1998; Ferrer et al., 2003; Hers, 1976; Agius, 2008). Glycogen phosphorylase-a is an allosteric inhibitor of

glycogen synthase phosphotase by binding to an allosteric site on GL (Pautsch et al., 2008).

1.3 Insulin action in liver

Insulin is a key hormone regulating glucose metabolism in many tissues, such as liver, the skeletal muscle and adipose tissue. The actions of insulin are uptake of glucose, amino acids transport, glycogen synthesis, protein synthesis and control of transcription of specific genes. However, some tissues have insulin-independent glucose transport, such as liver and cells of the nervous system (Baron and Van Obberghen, 1995). In liver, large members of genes are regulated at the transcriptional level by insulin (O'Brien et al., 2001).

1.3.1 Mechanisms of insulin signalling

The insulin receptor (IR) is a member of the tyrosine kinase receptor family (Goalstone and Draznin, 1997). The receptor is a tetrameric glycoprotein (190kDa). It contains two α-subunits (insulin binding domain) and two β-subunits (transmembrane; tyrosine kinase) held together by disuphide bonds (Baron and Van Obberghen, 1995). Insulin binding to α-subunits induces autophosphorylation of the tyrosine kinase activity in the β-subunits and phosphorylation of insulin receptor substrate (IRS) proteins (Saltiel and Kahn, 2001). After they are phosphorylated, IRS proteins interact with specific well-conserved domains, such as src-homology 2 domains (Goalstone et al., 1997). In addition, IRS proteins contact the plasma membrane by their pleckstrin homology domains. Phosphatidylinositol 3-kinase is a heterodimeric enzyme composed of a p110

catalytic subunit and a p85 regulatory subunit, which catalyzes the conversion of the lipid phoshatidylinositol Ptdlns(4, 5)P2 to Ptdlns(3, 4, 5)P3. Ptdlns(3, 4, 5)P3 functions as the key second messenger in insulin signalling (Fritsche et. al., 2008). Ptdlns(3,4,5)P3 regulates the localization of 3-phosphoinositide-dependent protein kinase 1 (PDK1), protein kinase B (PKB) or also called Akt and protein kinase C (PKC) through interaction with the PH (pleckstrin homology) domain (Saltiel and Kahn, 2001). The activation of PKB/Akt occurs rapidly upon insulin stimulation of the cell. There are many proteins in cytosol and nucleus that are regulated by PKB/Akt, and are involved in glycogen synthesis, proteins synthesis and control of gene transcription.

1.3.2 Insulin action on gene transcription

Insulin is involved in regulation of expression of mRNA and protein levels of several genes. However, the mechanisms are incompletely understood. The action of insulin could be positive or negative on the transcription and mRNA translation of gene expression. One of the main genes regulated by insulin in the liver is glucokinase (Iynedjian, 2009). Transcription factors that have been implicated in the control by insulin include SREBP-1c, HNF4-α, FOXO1 and HIF-1a. The role of SREBP-1c is controversial because although SREBP-1c is transcriptionally induced by insulin and also constitutive overexpression of SREBP-1c was shown to be associated with increased glucokinase expression (Foretz et al., 1999). Other studies showed that SREBP1c does not bind to the glucokinase promoter (Stoeckman and Towle, 2002) and also that activation of protein kinase B induces glucokinase expression in cells lacking SREBP-1c (Ono et al., 2003; Hansmannel et al., 2006). Although several studies have provided evidence for binding of

FOXO1 to the glucokinase promoter (Ganjam et al., 2009), normal insulin induction of glucokinase in FOXO1 deficient cells has also been reported (Matsumoto et al., 2007), suggesting involvement of other mechanisms. The induction by insulin of glucokinase is inhibited by glucagon through a mechanism involving cyclic adenosine monophosphate (cAMP) (Nouspikel and Iynedjian, 1992). Insulin also induces several enzymes of lipogenesis (including FAS) and the pentose phosphate pathway and it is accepted that the induction of these genes by insulin unlike the induction of glucokinase is mediated by SREBP-1c (Hansmannel et al., 2006; Towle, 2001),

Insulin represses several genes for example, phosphenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pc), insulin-like-growth-factor-binding protein 1 (IGFBP-1) and tyrosine aminotransferase (TAT). These genes are induced by glucagon through an increase in cAMP via CREB/CRTC2 (O'Brien et al., 2001). Transcription factors that are implicated in the regulation of gluconeogenic gene expression by insulin include FOXO1, FOXO3, HNF4α, C/EBP and SREBP-1c (O'Brien and Granner, 1996; Hutton and O'Brien, 2009).

1.3.3 The role of FOXO in insulin regulation of transcription

The FOXO's family of transcription factors was first identified by Weigel et al., 1989. There are more than 100 members of the FOX super family that are classified from A to R on the basis of sequence similarity (Myatt and Lam, 2007). The members of class O transcription factors include FOXO1 also known as Forkhead box 1 (FKHR), FOXO3A (FKHRL1), FOXO4 (AFX) and FOXO6 (Jacobs et al., 2003). These members of the FOXO family contain a conserved 100 amino acid domain, which is called winged helix DNA-binding domain. The

FOXO transcription factors are regulated by insulin and other growth factors (Barthel et al., 2005) and they control expression of target genes involved in metabolism, apoptosis and cells stress (van der Horst and Burgering, 2007; Wang et al., 2009). The FOXO family transcription factors are expressed in several tissues such as liver, skeletal muscle, adipose and others. The action of insulin signaling in hepatocytes, which is regulated through protein kinase B (PKB/Akt) causes phosphorylation of FOXO1 an multiple sites including Thr24 which causes translocation from nucleus to cytoplasm (Desvergne et al., 2006; Matsumoto et al., 2007; Qu et al., 2006; Naimi et al., 2007) (Fig. 1.1). FOXO1 plays an important role in glucose metabolism; its target genes included G6Pc, PEPCK, PGC-1α and IGFBP-1 in the liver (Bastie et al., 2005; Matsumoto et al., 2007; Barthel et al., 2005). In addition, the stimulation of expression of glucose 6-phosphatase (G6Pc) and phosphoenolpyruvate carboxykinase (PEPCK) are induced by FOXO1 and PGC1α in the fasting state. However, in the fed state, insulin activates Akt to phosphorylate FOXO1 and PGC1α resulting in translocation to the cytoplasm. On another hand, some studies have reported that there is regulation of FOXO1 by inducing oxidative stress which is activated through various post-translational modifications including phosphrylation, acetylation and ubiquitination (Zhao Y, et al. 2010; Ponugoti B, et al. 2012). It seems that FOXO1 plays an important role in protection of cells from oxidative stress which is caused by glucose. Previous studies have reported that the FOXO1 and FOXO3A transcription factors have been shown to bind the Thioredoxin interacting protein (TXNIP) promoter and regulate the expression of TXNIP in hepatocytes (Yu and Luo, 2009; Zhuo et al., 2010).

1.4 Glucose action on gene transcription

Several studies have shown that high glucose concentration regulates gene transcription (Girard et al., 1997; Massillon, 2001; Dentin et al., 2012). This effect can be mediated either by an effect of glucose metabolism on insulin signalling or by an effect on transcription factors that are either regulated by insulin signalling or by other signals. Two general areas of research have emerged on glucose regulation of gene transcription: the study of the covalent modification of proteins involved in insulin signalling (e.g. IRS1, Akt) or gene regulation (FOXO1) by covalent modification by O-GlcNAc and the study of transcription factors that are targets of glucose metabolism (e.g. ChREBP, MondoA, LXR). Studies on animal models fed a high carbohydrate diet identified enzymes of glycolysis and lipogenesis as target genes for both insulin and glucose through independent regulatory elements on the promoter. The regulatory element that binds SREBP-1c is termed the sterol regulatory element and it mediates regulation by insulin whereas the DNA element that is regulated by glucose is termed the carbohydrate response element (ChoRE) (Towle, 2001). Studies on the mechanism by which glucose regulates gene expression in both liver and adipose tissue showed that metabolic conditions that lead to an increase in glucose 6-P cause the induction of several genes and this led to the suggestion that glucose 6-P is the active metabolite for gene expression (Girard et al., 1997). However, other studies on liver derived cell lines suggested a metabolite of the pentose phosphate pathway is most likely involved (Kahn, 1997) and based on experiments with xylitol which causes a similar or larger induction of gene expression than glucose a role for xylulose 5-P was suggested (Kabashima et al., 2003; Dentin et al., 2012; Uyeda and Repa, 2006). G6Pc catalyses the final reaction in hepatic glucose production and was also shown to be regulated by xylitol by a mechanism most

likely involving xylulose 5-P (Massillon, 2001). In non-hepatic cells, and in skeletal muscle of subjects with type 2 diabetes, TXNIP was identified as a major glucose inducible gene (Parikh et al., 2007). TXNIP is also induced by high glucose in hepatocytes (Ma et al., 2006).

1.4.1 The role of ChREBP in glucose regulation of transcription

The transcription factor Carbohydrate response element binding protein (ChREBP) has a key role linking glucose metabolism and control of gene expression of enzymes of glycolyis and lipogenesis. ChREBP is a large transcription factor, approximately 850 amino acid residues with a molecular weight of 100 kDa. ChREBP was discovered independently by two research groups. Cairo group's showed that the ChREBP gene (WBSCR14) is located in a DNA region that is mutated in patients with the developmental disorder Williams-beuren Syndrome (Cairo et al., 2001). They showed expression of ChREBP at mRNA level by in situ hybridization or Northern Blot in various tissues including liver, gut and brain and that it forms heterodimers with the protein Mlx. They also showed that ChREBP protein has a short half-life when transfected in Cos cells. Mlx is a member of the Myc/Max/Mad family of transcription factors with a basic helix-loop-helix leucine zipper (bHLHZip) domain required for DNA binding by the resulting heterocomplex (Peterson and Ayer, 2011). Mlx can serve as partner of various transcriptional regulators including Mad1, Mad4 and Mnt which function as repressors.

Uyeda group's purified ChREBP by using the proximal promoter region of the pyruvate kinase gene to search for the transcription factor that regulates gene expression of enzymes of glycolysis and lipogenesis in animals fed a high

carbohydrate diet (Kawaguchi et al., 2001). They showed that ChREBP is expressed at high levels in liver and adipose tissue and that expression of ChREBP in liver is increased in animals fed a high carbohydrate diet (Uyeda et al., 2002). They proposed a major role for ChREBP in mediating the effect of high glucose on gene expression of enzymes of glycolysis and lipogenesis because ChREBP knock-out mice have a decreased induction of enzymes of lipogenesis in response to high-carbohydrate diets in vivo and also a decreased response of isolated hepatocytes from these animal models to glucose induction of gene expression (Iizuka et al., 2004). Uyeda also showed that knock-down of ChREBP in the ob/ob mouse also down regulates the expression of lipogenesis in this model, suggesting that the induction of lipogenesis in an animal model of insulin resistance is also due to ChREBP (Iizuka et al., 2006). Similar results were obtained by other research groups who showed that down regulation of ChREBP in liver with sh-adenoviral vectors also decreases the expression of enzymes of lipogenesis and development of fatty liver in this model (Dentin et al., 2006). The suggested function of ChREBP from these studies is that it regulates the conversion of carbohydrate to fat on a high carbohydrate diet and also in models of hyperphagia and insulin resistance (Uyeda and Repa, 2006, Towle et al., 1997). Studies by Stoeckman and colleagues showed that ChREBP binds to the promoter of glucose responsive genes in the liver as a heterodimer with Mlx when hepatocytes are incubated at high glucose concentration. In earlier studies they identified the glucose response element of carbohydrate responsive genes (Stoeckman et al., 2004) as consisting of two imperfect E-boxes separated by 5 bases. Ma and colleagues then used a dominant negative variant of Mlx to identify new genes that are regulated by glucose through an Mlx-dependent mechanism. By using gene microarray studies they identified large numbers of genes that are

either induced or repressed by high glucose through an Mlx-dependent mechanism either directly or indirectly (Ma et al., 2006). The genes induced by glucose included not only enzymes of glycolysis and lipogenesis but also G6Pc and fructose 1,6-bisphosphatase. However, whether these genes are direct or indirect targets of ChREBP-Mlx was not determined.

1.4.2 Mechanism of activation of ChREBP by glucose metabolism

The mechanism by which glucose signalling activates ChREBP is incompletely understood. Uyeda and colleagues proposed that phosphorlyation of ChREBP on residues Ser(196) and Thr(666) by glucagon through activation of cAMP dependent protein causes accumulation of ChREBP in the cytoplasm, and high glucose cause dephosphorylation of these residues and translocation of ChREBP to the nucleus and binding to DNA and for activation of gene transcription (Fig. 1.2). Uyeda proposed that metabolism of glucose by the pentose phosphate pathway causes an increase in xylulose 5-P, which causes activation of the protein phosphatase 2A (PP2A) to dephosphorylate ChREBP and promote its nuclear localization and DNA binding (Kabashima et al., 2003; Dentin et al., 2012; Uyeda and Repa, 2006). However, other studies found that mutation of these residues did not affect the stimulation by glucose of translocation of ChREBP to the nucleus (Tsatsos and Towle, 2006). Studies by Li and colleagues proposed activation of ChREBP by binding of glucose or glucose 6-P to the N-terminus of ChREBP (Li et al., 2010). These studies used islet beta cells and expression of a GAL4-ChREBP fusion protein that is targeted constitutively to the nucleus and also a luciferase reporter containing ChoRE elements to study the metabolites of glucose that activate transcription by GAL4-ChREBP. These studies showed that the

stimulation by high glucose was inhibited with a glucokinase inhibitor and also by overexpression of PFK1 and PFK2 and suggested that the stimulation was by glucose 6-P. They also showed a small activation by 2-deoxyglucose and concluded that activation of ChREBP after DNA binding is explained by glucose 6-P. Dentin and colleagues studied the metabolite mechanism for ChREBP translocation, DNA binding and activation of transcription and proposed that the mechanism is not mediated by xylulose 5-P but by glucose 6-P (Dentin et al., 2012), because changes in gene transcription correlated with changes in glucose 6-P.

1.4.3 Enzymes regulated conversely by glucose and insulin

1.4.3.1 The role of glucose 6-phosphatase (G6Pc)

Glucose 6-phosphatase (G6Pc or G6Pc1) is an important enzyme in glucose production. It catalyzes the conversion of glucose 6-P to glucose the last step of glucose production by glycogenolysis and gluconeogenesis in the liver (Hutton and O'Brien, 2009). G6Pc protein consists of 357 amino acid with a molecular weight of approximately 36 kDa in liver (van de Werve et al., 2000). G6Pc is located inside the lumen of the endoplasmic reticulum with the catalytic site facing the lumen. Function of G6Pc is coupled to a transporter protein encoded by SLC37A4 which transports glucose 6-P from the cytoplasm to the lumen. The activity of G6Pc was higher on glucose 6-P rather than other substrates phosphate such as mannose 6-P, glucosamine 6-phosphate (GlcN 6-P) and 2-deoxyglucose 6-phosphate (2-DOG 6-P) which catalyses specifically the hydrolysis of glucose 6-P (Hutton and O'Brien, 2009).

There are three isoforms of the G6Pc gene family called G6Pc 1, 2 and 3. The liver isoform known as G6Pc or G6Pc1 is expressed in hepatocytes, nephrocytes, enterocytes and in β-cells (Ven Schaftingen and Gerin, 2002). G6Pc2 is expressed in islet beta cells and G6Pc3 is expressed in all tissues. In the kidney G6Pc1 also has a role in glucose production and during fasting and diabetes it is thought to contribute to glucose production by up to 25%. During liver transplantation glucose production by the kidney is increased to compensate for the lack of contribution by the liver (Joseph et al., 2000). In the small intestine the suggested functions of G6Pc1 are in glucose transport and conversion of fructose to glucose (Bismut et al., 1993; Cui et al., 2004; Suzuki et al., 2011). Whereas in the islet beta cells the suggested function of G6Pc1 and also G6Pc2 are as negative regulators of insulin secretion (Dos Santos et al., 2009). Although the major function of G6Pc1 is the production of glucose, another function in the liver is to maintain intracellular homeostasis of glucose 6-P by preventing excessive elevation in glucose 6-P (Aiston et al., 1999). The main evidence for this function comes from studies involving inhibitors of the G6PT, such as the chlorogenic derivative S4048. This inhibitor has little effect on the hepatocyte glucose 6-P concentration at 5mM glucose but it causes a very large increase in glucose 6-P accumulation at concentrations above 10mM (Harndahl et al., 2006). This supports an important function of G6Pc in the liver cell in homeostasis of intracellular glucose 6-P (Agius, 2013).

In the liver the expression of G6Pc at both the mRNA levels and the activity is increased by glucose, glucagon, glucocorticoids and fatty acids and is decreased by insulin. The proximal G6Pc promoter region in the liver binds several transcription factors including FOXO1, HNF1, the glucocorticoid receptor, Foxa2,

HNF6, HNF4α, CREB and Sp1/Sp3. (Ayala et al., 1999; Barthel et al., 2001; Schmoll et al., 2000).

1.4.3.2 The role of Thioredoxin interaction protein (TXNIP)

TXNIP was first identified in 1993 and is also known as vitamin D_3 up-regulated protein 1 (VDUP-1) and thioredoxin binding protein 2 (TBP2) and is a binding protein of thioredoxin (Chutkow et al., 2008). TXNIP is a 46kDa protein and member to a family of six structurally similar arrestin domain-containing proteins (ARRDC) also called α-arrestins. Thioredoxin (TRX) is a small (12kDa) multifunctional protein consisting of 105 amino acids and exists as many isoforms, such as TRX 1, the cytosolic (classical member) and TRX 2, the mitochondria-specific type, with a conserved catalytic site acting as a potent antioxidant protein reducing oxidized protein and preventing accumulation of excess reactive oxygen species (ROS) which protect cells from damage (Kaimul et al,. 2007). The function of thioredoxin is to transfer electrons from NADPH to the disulphide groups of proteins causing their reduction. TXNIP functions as an inhibitor of thioredoxin and the ratio of thioredoxin-to-TXNIP is thought to determine the redox state of –SH groups in proteins and reduce sulfhydryl groups (Fig. 1.3). There are two ways that the function and action of TRX can be inhibited by TXNIP: (i) TXNIP binds TRX and acts as a competitive inhibitor to remove TRX from proteins whose function is inhibited by the steric effect of TRX binding such as apoptosis signal regulating kinase 1. (ii) Increases in TXNIP expression by factors such as high glucose (Stoltzman et al., 2008; Cha-Molstad et al., 2009; Yu and Luo, 2009), various stress stimuli including H_2O_2, TGF-β, UV and heat shock (Junn et al., 2000; Han et al., 2003; Kim et al., 2004), the histone

deacetylase (HDAC) inhibitors (Butler et al., 2002) and NAD+ (Yu and Luo, 2009) result in reduced TRX reductase activity.

The increase in TXNIP-TRX complexes will result in more oxidized proteins on exposure to oxidative stress. There are several functions of TXNIP including regulation of glucose uptake and cell growth and apoptosis (Kaadige et al., 2009, Peterson et al., 2010, Kaadige et al., 2010, Chutkow et al., 2008). TXNIP has therefore been proposed to have a role in causing apoptosis in islet β-cells (Kaadige et al., 2009, Peterson et al., 2010, Kaadige et al., 2010, Chutkow et al., 2008). Through the inhibition of TRX, TXNIP overexpression enhances β-cell death and impairs insulin secretion (Minn et al., 2005; Chen et al., 2006).

TXNIP is a key regulator of glucose metabolism and insulin signaling and therefore controls cellular energy status. Examples of enzymes which are very sensitive to the redox state of their thiol groups include phosphatases with sequence homology to tensin (PTEN) which is a negative regulator of insulin action (Akt downstream signaling) (Hui et al., 2008). Increasing Thiol redox status regulates enzyme involved in gluconeogenesis such as hepatic fructose-1,6-bisphosphatase which is negatively regulated by fructose 2,6-bisphosphate (Hui et al., 2004). TXNIP overexpression inhibits Akt/PKB signalling (Shaw and Cantley, 2006; Peterson and Ayer, 2011) and causes accumulation of transcription factors such as FOXO1 and FOXO3A in the nucleus. Furthermore, bioinformatics studies showed that TXNIP has a FOXO binding site which suggested regulation between FOXO and TXNIP (Fa-Xing and Luo, 2009). In pancreatic β-cells the induction of TXNIP mRNA gene expression by high glucose involves ChREBP, and has a role in promoting apoptosis (Minn, et al. 2005). In several non-hepatic cell lines, high glucose induces TXNIP by MondoA (Peterson and Ayer, 2011). A recent study has shown that MondoA was more active than ChREBP in regulating

TXNIP by comparing the activity of MondoA and ChREBP at the TXNIP promoter using a luciferase-based reporter system in a Hela cell line (Yu et al., 2009).

Some research studies showed that high glucose causes an increase in ROS and this increases TXNIP mRNA expression (Qi et al., 2007) or ROS activates TXNIP by activation of MAPK signalling (Fang et al., 2011). Reactive oxygen species (ROS) or oxidative stress is a pathogenic factor of hyperglycemia and diabetes complication. The major sources of ROS production on biological systems were found in the mitochondria and NAD (PH) oxidase (Valko et al., 2007). The increase of ROS in biological system causes damage of DNA, lipids, membranes and proteins.

1.5 The Hexosamine Biosynthesis Pathway (HBP)

Several studies have proposed that high glucose concentration causes insulin resistance through increased flux through the hexosamine biosynthesis pathway (HBP) and that this pathway contributes to the glucose toxicity of diabetes (Whelan et al., 2008; Yang et al., 2008; Vosseller et al., 2002; Arias, 2004).

The first reaction of this pathway is the synthesis of glucosamine 6-P from fructose 6-P and glutamine catalysed by the enzyme glutamine:fructose 6-phosphate amidotransferase (GFAT) the rate-limiting step of the HBP from glucose (Copeland et al., 2008; Love and Hanover, 2005). The concentration of glucose 6-P in the liver is related to the extracellular glucose concentration and also to the activities of glucokinase and glucose 6-phosphatase (Agius et al., 2008). Glucose 6-P is in equilibrium with fructose 6-P, the first intermediate of the glycolytic pathway. Fructose 6-phosphate in addition to being an intermediate

of glycolysis is also a substrate for the hexosamine biosynthesis pathway (HBP). An alternative route for synthesis of glucosamine 6-P is from glucosamine which can be phosphorylated by glucokinase or other hexokinases. Glucosamine is often used in experimental studies to increase flux through the HBP. The glucosamine 6-phosphate is converted through a sequence of reactions into uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) and this is a substrate for various pathways of glycosylation of proteins which include N-GlcNAcylation and O-GlcNAcylation modification of protein (Copeland et al., 2008; McClain and Crook, 1996; Love and Hanover, 2005) (Fig. 1.4).

The O-linked beta-N-acetylglucosamine (O-GlcNAc) transferase (OGT) catalyses the transfer of O-GlcNAc to serine and threonine residues (Copeland et al., 2008; Wang et al., 2009). This reaction is reversed by the enzyme Beta-N-acetylglucosaminidase (OGA or also known as O-GlcNAcase) which catalyses the removal of the O-GlcNAc group. The role of O-linked glycosylation of proteins has been studied using inhibitors of OGT and OGA and various studies have suggested a role for this covalent modification in the regulation of insulin action. Several studies have suggested that O-linked glycosylation plays a key role in insulin resistance by regulating gene expression, insulin signalling and glucose uptake (Buse, 2006; Copeland et al., 2008; Love and Hanover, 2005).

Three different experimental approaches have been used to study the function of the hexosamine biosynthesis pathway (HBP). One method involves use of the GFAT inhibitors azaserine (AZN) and 6-diazo-5-oxonorleucine (DON), to block the metabolism of glucose by the HBP (Zachara and Hart, 2006; McClain and Crook, 1996). Another involves incubation with glucosamine which is converted to glucosamine 6-phosphate. Glucosamine enters the HBP after GFAT and leads to increased production of glucosamine 6-phosphate because it by passes the rate

limiting step. A third approach involves overexpression of GFAT. There are two isoforms of GFAT; GFAT1 is expressed ubiquitously, whereas GFAT2 is expressed in liver. The first purification of GFAT from rat liver was described by Pogell and Gryder in 1957 (Huynh et al., 2000). GFAT1 and GFAT2 differ in their kinetic and regulatory properties (Ye et al., 2004; Zhou et al., 1998; Oki, et al., 1999; Hu et al., 2004).

1.5.1 The enzymes of O-GlcNAc metabolism (OGT and OGA)

There are three isoforms of OGT, the 110 kDa and 78kDa forms are found in the nucleus as heterotrimers (nOGT) and the 103 kDa is found in the mitochondria (mOGT) (Hurtado-Guerrero et al., 2008). The domain structures of OGT are an N-terminal tetratricopeptide repeat (TPR) domain and a C-terminal catalytic domain (Zachara and Hart, 2006). There are two isoforms of OGA, a 130 kDa and a 75 kDa. OGA comprises an N-terminal hexosaminidase domain and a putative C-terminal histone acetyltransferase (HAT) domain (Rexach et al., 2008; Zachara and Hart, 2006). O-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino N-phenylcarbamate (PUGNAc) is inhibitor of OGA, which causes an increase in O-linked glycosylation levels and resulted in the establishment of insulin resistance (Copeland et al., 2008). Inhibitors of OGA (PUGNAc) as of OGT (BADGP) have been ued to study the role of O-GlcNAc modification of proteins in insulin action, and insulin resistance. These studies have also used glucosamine at concentrations up to 10 mM to increase covalent modification of protein by O-GlcNAc, which was shown by western blotting using an antibody against O-GlcNAc (D'Alessandris et al., 2004; Copeland et al., 2008). In addition, streptozotocin (STZ) is another inhibitor of OGA (Li et al., 2012). However, some studies have

reported that STZ is a poor inhibitor of OGA and there are many side effects (toxic to cells) (Copeland et al., 2008).

1.5.2 Covalent modification by O-GlcNAc in insulin signaling

Increased O-GlcNAc modification by overexpression of OGT or inhibition of O-GlcNAcase (OGA) with PUGNAc in 3T3-L1 adipocytes and skeletal muscle caused insulin signalling pathway dysfunction (Vosseller et al., 2002; Matthew, 2008). In addition, treatment of 3T3-L1 adipocytes with PUGNAc which increased O-linked glycosylation decreased insulin stimulation of 2-deoxyglucose uptake but did not affect IRS protein tyrosine phosphorylation by insulin suggesting that glycosylation affects downstream sites in insulin signalling (Vosseller et al., 2002). However, another study has reported that PUGNAc did not affect insulin-stimulated phosphorylation of Akt or glycogen synthase kinase-3 (GSK-3) in the skeletal muscle (Arias et al., 2004). Furthermore, some studies showed that OGT has an Ptdlns(3, 4, 5)P3 binding domain and that insulin signalling causes translocation of OGT from cytoplasm to nucleus which causes covalent modification of Akt. They also showed that increased O-GlcNAc modification by overexpression of OGT caused a decrease the phosphorylation of Akt at Thr 308 and increase phosphorylation of IRS1 at Ser 307 and Ser 632/635 (Yang et al., 2008).

1.5.3 Covalent modification of transcription factors by O-GlcNAc

Covalent modification of transcription factors by O-GlcNAc affects the location and activity by different mechanisms including:: (i) by controlling the length of

time transcription factors spend in the nucleus; (ii) by targeting transcription factors for proteolytic degradation; (iii) by regulating transcription factors DNA binding; (iv) by complex formation between transcription factors (modulating protein-protein interactions) and (v) by modifying chromatin structure (Whitmarsh and Davis, 2000).

Two groups suggested an effect of O-GlcNAc modification on ChREBP protein by high glucose. Sakiyama H, et al. 2010 showed that inhibitors of OGA caused an increased effect of glucose and inhibition of GFAT caused a decreased effect of glucose on ChREBP activation of gene expression measured with a luciferase reporter assay (Sakiyama et al., 2010). Guinez and colleagues reported that OGT binds to ChREBP and that animal models of hyperglycaemia show increased O-GlcNAc modification of ChREBP which results in its stabilisation resulting in increased protein level of ChREBP in liver (Guinez et al., 2011).

Other studies have reported that O-GlcNAc glycosylation of FOXO1 caused by glucose, GlcN and PUGNAc resulted in the induction of G6Pc and PGC1α mRNA levels (Kuo M, et al. 2008). Studies by Housley and colleagues show that high glucose causes O-GlcNAc modification of FOXO1 in the absence of insulin which induces G6Pc and PEPCK mRNA levels (Housley et al., 2008). Therefore, O-GlcNAc modification of FOXO1 increased expression of G6Pc gene in glucotoxicity in diabetes and obesity (Kuo et al., 2008; Housley et al., 2008; Housley et al., 2009; Ido-Kitamura et al., 2012).

1.5.4 Endoplasmic reticulum (ER) stress and the HBP

The endoplasmic reticulum (ER) is a central organelle which synthesizes various proteins secretory products. Several pathways are integrated to regulate lipid,

glucose, cholesterol and protein metabolism (Sundar Rajan, S. et al. 2007; Yoshida H. 2007; Gregor, MF. et al. 2007). ER is a major site of protein synthesis and folding. Ribosomes attached to the ER translate peptides into the luminal space of the ER. In the ER luminal protein chaperones such as BiP/GRP78, calnexin and calreticulin help folding and prevent aggregation of unfolded or misfolded proteins. Folded proteins released into the Golgi apparatus for transport to cellular destination or secretion (Gregor, MF. et al. 2007). In addition, other functions of the ER are site of triglyceride synthesis and assembly of lipid droplet, cholesterol sensing and sequestration of the sterol-regulatory element binding protein (SREBP-1c), which is released in response to low cholesterol or insulin signalling and accumulation of calcium which plays a role in cellular calcium homeostasis (Gregor, MF. et al. 2007).

Various studies have shown that compounds that alter either the flux through the HBP or protein glycosylation (glucosamine, tunicamycin and other glycosylation inhibitors) cause ER stress. Tunicamycin and 2-deoxy-D-glucose are inhibitors of N-glycosylation but 2-deoxy-D-glucose is less efficient than tunicamycin (Yoshida H. 2007; Werstuck, GH. et. al. 2006; Kim, AJ. et. al. 2004).

Changes in glycosylation of proteins can lead to abnormal accumulation of proteins in the ER which stimulates the unfolded protein response (UPR) (Kim, AJ. et. al. 2004). For example, Chaperone protein such as BiP normally binding to three transmembrane proteins such as eukaryotic initiation factor 2α kinase (PERK), inositol-requiring enzyme-1 (IRE-1) and activating transcription factor-6 (ATF-6) which causes stress sensing proteins inactive. The larger excess of unfolded proteins or accumulation (also bound by BiP) made less BiP available for binding to the unfolded protein response (UPR) sensors. BiP (chaperone) dissociates from PERK, IRE-1 and ATF-6, which leads to the activation of many

signalling pathways (Gregor, MF. et al. 2007; Sundar Rajan, S. et al. 2007; Robertson, LA. et. al. 2006). The activation of PERK, IRE-1 and ATF-6 causes phosphorylation of eukaryotic translational initiation factor 2α (elf2α) which inhibits protein synthesis, phosphorylation of IRE-1 activates JNK and NFkB and ATF-6 is released to the Golgi where it becomes an active transcription factor and translocates to the nucleus and induces protein chaperone expression (Gregor, MF. et al. 2007; Sundar Rajan, S. et al. 2007).

1.6 Hypothesis and aims of project

Type 2 diabetes is associated with increased hepatic and peripheral insulin resistance. The hepatic insulin resistance is associated with increased lipogenesis and glucose production. The increased hepatic lipogenesis is generally explained by the induction of enzymes of glycolysis and lipogenesis by high glucose concentration through activation of ChREBP (Towle, 2005). The increased gluconeogenesis, which is associated with increased expression of the enzyme glucose 6-phosphatase (G6Pc), is explained covalent modification of transcription factors by O-GlcNAc as a result of increased flux through the hexosamine biosynthesis pathway (HBP). G6Pc is induced by glucagon and repressed by insulin and transcription factors that are regulated by these hormones are implicated in the covalent modification by O-GlcNAc (Housley et al., 2008, Kuo et al., 2008, Dentin et al., 2008). Insulin resistance in extrahepatic tissues is associated with increased expression of TXNIP which is also induced by high glucose (Parikh et al., 2007) and is a target gene for FOXO transcription factors. There is little information on flux through the HBP in liver or whether glucose regulation of gene expression correlates with changes in HBP flux.

This thesis tested the hypothesis that gene expression of G6Pc and TXNIP in hepatocytes is regulated by the glucose-activated transcription factor ChREBP-Mlx and by flux through the HBP through covalent modification of transcription factors.

The aims of the thesis were: first, to develop methods for measurement of flux through the HBP in primary hepatocytes and to validate experimental tools to change flux through the HBP, to establish the role of the pathway in glucose regulation of gene expression; second, to determine whether glucose regulation of G6Pc occurs through the glucose-regulated transcription factor ChREBP or through covalent modification by O-GlcNAc; third to compare the regulation by glucose of G6Pc with TXNIP which represent major glucose-inducible genes in insulin resistance in liver and peripheral tissues, respectively.

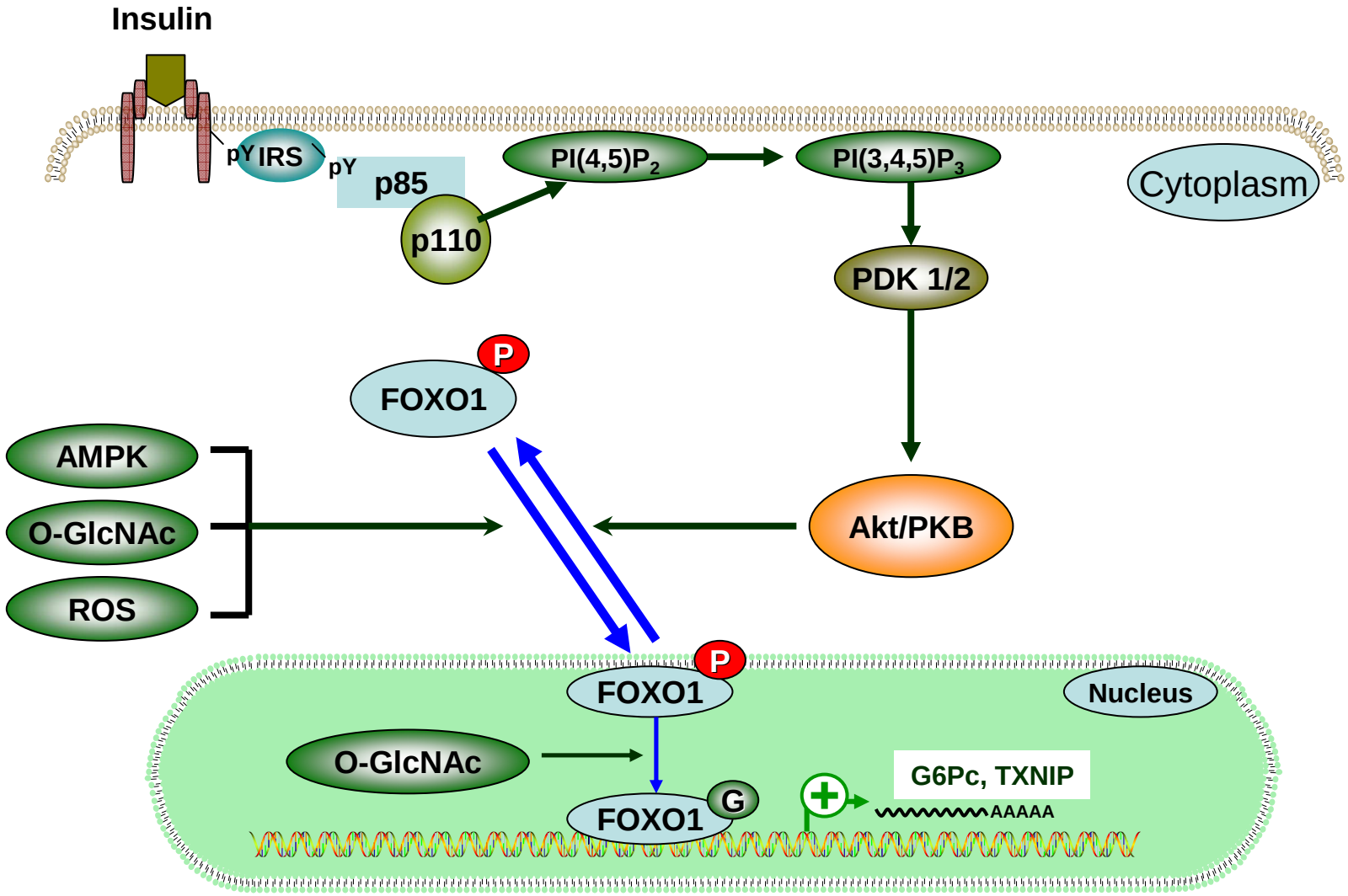


Fig. 1.1 Regulation of Akt/PKB causes phosphorylation of FOXO1 and causes translocation from nucleus to cytoplasm

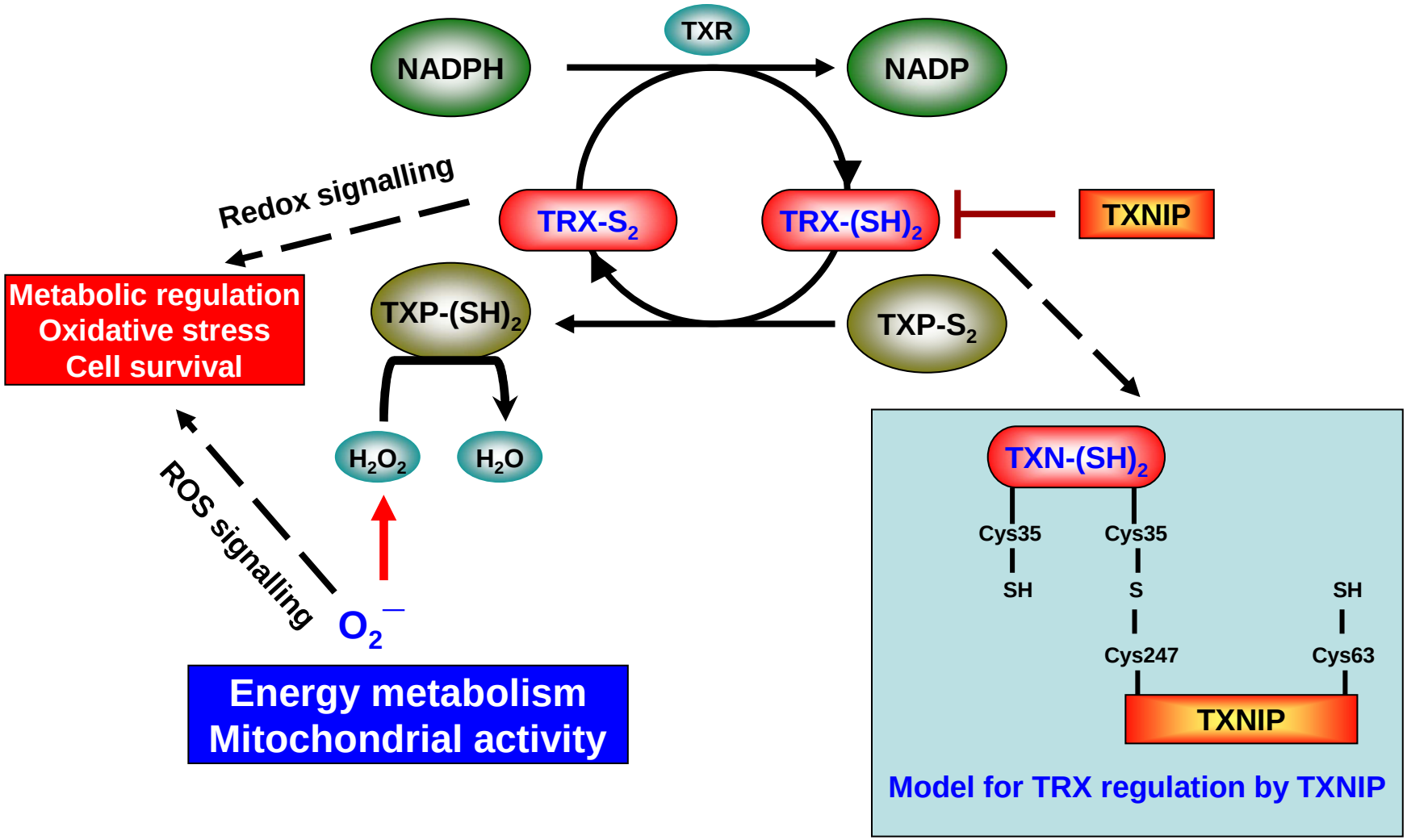


Fig 1.3 The action of TXNIP on Thioredoxin (TRX) (Muoio, DM. 2007)

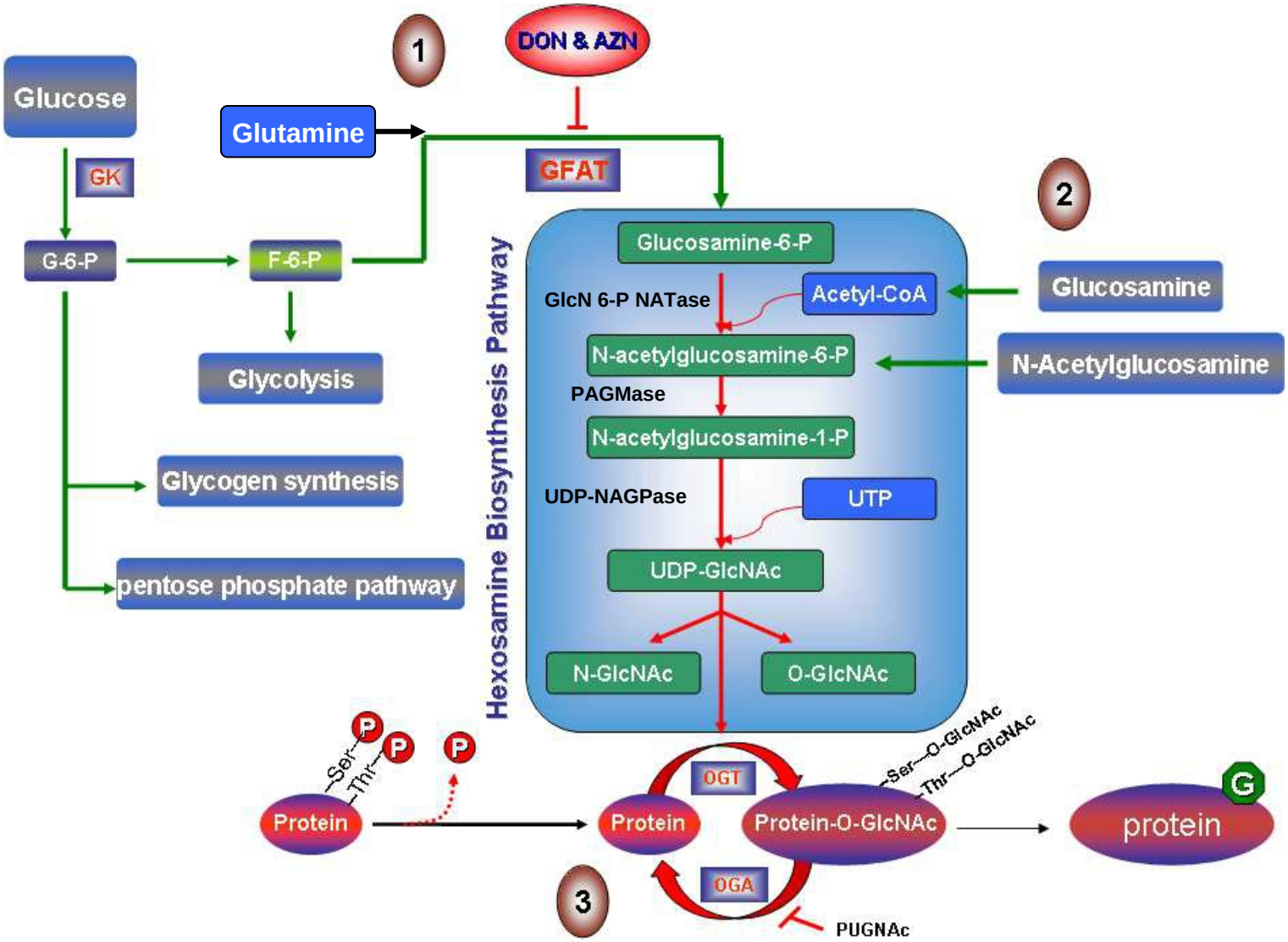


Fig. 1.4 The Hexosamine Biosynthesis pathway (HBP) and covalent modification of protein by O-GlcNAc
Flux through the HBP can be studied using: (i) selective GAFT inhibitors (DON and AZN); (ii) glucosamine (which bypasses GFAT); (iii) OGT and OGA inhibitors or overexpression of OGT

Chapter 2

Materials and Method

2.1 Materials

2.1.1 Animals

Male albino Wister rats, of the Hanover strain (body weight 180-380 g), were obtained from either B&K Universal Ltd (Hull, UK) or from Harlan Bicester, UK. They were subjected to light-dark cycles every 12 h with free access to water and fed ad libitum on standard laboratory chow.

2.1.2 Chemicals and Reagents

Buffer and common salts were obtained from BDH Laboratory Supplies (Poole, Dorset, UK). Commercial chemicals and reagents were purchased from manufacturers indicated in table 2-1.

Chemicals/Reagents	**Supplier**	**Cat. No.**
Eagles' Minimum Essential Medium (X1)	Gibco, Invitrogen	32360
Non-essential amino acids (100x)	Gibco, Invitrogen	11140-035
Neonatal bovine serum	Gibco, Invitrogen	26010-074
Streptomycin sulphate	Sigma	S0130
Tunicamycin	Sigma	T7765
Benzamidine	Sigma	B-6506
Phenyl methanesulfonyl fluoride	Sigma	P7626
Penicillin G sodium salt	Sigma	P3032
4-Dimethylaminobenzaldehyde	Sigma	15,647-7
Glucose 6-phosphate	Sigma	P7879
2-Deoxy-D-glucose	Sigma	D8375

Xylitol	Sigma	X3375
3-O-Methyl-D-glucopyranose	Sigma	M4879
N-acetyl-D-glucosamine	Sigma	A8625
Allose	Sigma	285005
D-Glucosamine	Sigma	G-4875
5-Thioglucose	Sigma	88635
O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) N-phenylcarbamate (PUGNAc)	Tocris Bioscience	A7229
6-Diazo-5-oxo-L-norleucine (DON)	Sigma	D2141
Benzyl2-acetamido-2-deoxy-a-D-galactopyranoside (BADGP)	Sigma	B4894
Azaserine	Sigma	A4142
[U-^{14}C]glucose	PerkinElmer	NEC042V250UC
[2-^{3}H]glucose	PerkinElmer	NEC238C001MC
^{3}H-leucine	PerkinElmer	NET1166001MC
D-glucosamine-1-^{14}C	Sigma	G2770
N-acetylglucosamine-1-^{14}C	Sigma	311537
Actinomycin D	Sigma	A1410
Bromopyruvic acid	Sigma	16490
Verapamil	Sigma	PHR1131
PD 169319	Sigma	P9248
Paraformaldehyde	Sigma	P6148
Trizol	Sigma	15596-018
Primer random p(dN)$_6$	Roche	11034731001

(ECL) detection kit	Thermo Scientific	32106
(ECL) detection kit Plus	GE heathecare Ltd	32106
2-Bromo-2-chloro-1,1,1-trifluoroethane	Sigma	B4388
Gelatine	Sigma	G7041
BSA	Sigma	A7906
dNTP mix	Promega	U1240
LightCycler Faststart DNA Master SYBR	Roche	3003230
PMSF	Sigma	P7626
Protease inhibitor cocktail	Sigma	P8340
ChIP assay kit protocol	Millipore	17-295
Lectin from Triticum vulgaris-Agarose	Sigma	L1882
Phenol/chloroform/isoamyl alcohol mix	Sigma	77617
S4048	Gift from Dr. D. Schmoll, Aventis, Pharma GnbH, Frankfurt, Germany	

2.1.3 Enzymes

Commercial enzymes were purchased from manufacturers indicated in table 2-2

Enzymes	**Source**	**Cat. No.**
Collagenase	Sigma	C5138
Glucose 6-phosphate dehydrogenase (G6PDH)	Sigma	G8404
Phosphoglucomutase (PGM)	Sigma	P3397
Insulin	Sigma	I5523
Diaphorase	Sigma	D5540
Aldolase	Sigma	A8811
Glycerophosphate dehydrogenase	Sigma	G6880
Triosephosphate isomerase (TPI)	Sigma	T6258
Superscript II reverse transcriptase	Invitrogen	18064-071

RNase free DNase I	Roche	10776785001
RNasin Plus RNase inhibitor	Promega	N2615
Proteinase K	Sigma	P4850
M-MLV reverse transcriptase	Invitrogen	28025-021

2.1.4 Antibodies

Commercial antibodies were purchased from manufacturers indicated in table 2-3

Antibodies	**Host (Applications)**	**Supplier**	**Cat. No.**
FOXO1 (C29H4)	Rabbit (WB, IF)	Cell signaling	2880
FOXO3A (75D8)	Rabbit (WB, IF)	Cell signaling	2497
Phospho-FOXO1 (Ser256)	Rabbit (WB)	Cell signaling	9461
Phospho-FOXO1 (Thr24) /FOXO3A (Thr32)	Rabbit (WB)	Cell signaling	9464
Phospho-Akt (Ser473)	Rabbit (WB)	Cell signaling	9271
O-GlcNAc (CTD 110.6)	Mouse (WB)	Covance	MMS-248R
O-GlcNAc (RL2)	Mouse (WB)	Thermo Scientific	MA1-072
TXNIP	Mouse (WB, IF)	MBL	K0205-3
OGT (DM-17)	Rabbit (WB)	Sigma	O6014
Mlx	Goat (ChIP)	Santa cruz	Sc-14705
FKHR/FOXO1 (H-128)	Rabbit (ChIP)	Santa cruz	Sc-11350
FKHR/FOXO3A (H-144)	Rabbit (ChIP)	Santa cruz	Sc-11351
ChREBP	Rabbit (WB, ChIP)	Novus	NB-400-135
MondoA	Rabbit (ChIP)	Sigma	SAB2104303

Anti-acetyl-Histone H4	Rabbit (ChIP)	Merck Millipore	06-866
NF-Y	Rabbit (ChIP)	AbD serotec	AHP298
IgG	Rabbit (ChIP)	Santa cruz	Sc-2027
GAPDH	Mouse (WB)	Hytest	5G4
SREBP1c (2A4)	Mouse (WB, IF)	Abcam	Ab3259
Mouse immunoglobulins HRP	Mouse (WB)	Dako	P0260
Polyclonal goat anti-rabbit immunoglobulins HRP	Rabbit (WB)	Dako	Po448
Anti-mouse IgM H&L (goat) POD	Mouse (WB)	Calbiochem	401225
Alexa fluor 488 goat anti-rabbit IgG	Rabbit (IF)	Invitrogen	A11008
Alexa fluor 488 donkey anti-mouse IgG	Mouse (IF)	Invitrogen	A12102

2.1.5 Adenoviral vectors

Commercial adenoviral vectors were purchased from manufacturers indicated in table 2-4

Adenoviral vectors	**Source**
Glucokinase adenovirus	(Becker et al., 1996)
ChREBP/WT, Mlx/WT, MondoA/WT and Mlx dominant negative (Mlx-DN)	Gifts from H.Towle (Stoeckman et al., 2004)
PFK/WT and PFK-KD	Gifts from A. Lange (Arden et al., 2008)
FOXO3A/WT	(Cat.No. 1576) Vector biolabs the gene delivery company

CREB1/WT	(Cat.No. 1363) Vector biolabs the gene delivery company
Pre-Made Adenovirus-OGT	(Cat.No. 000619A) abm Applied biological materials inc.

Each batch was tested for overexpression in hepatocytes and lysates were aliquoted and stored at -80°C.

2.1.6 Primers for Real time RT-PCR

Primers were designed using the online Roche Universal ProbeLibrary Assay Design tool. Primers were synthesised by MWG Operon Biotech Ltd (Milton Keynes, UK) (table 2-5).

	Primers
ChREBP *(Mlxipl)*	FWD: GGGACATGTTTGATGACTATGTC REV: AATAAAGGTCGGATGAGGATGCT
DEC1 *(Dec1)*	FWD: CGTGAAAGCATTGACAAACC REV: TTCTTTCCCGACAGATCACC
FAS *(Fasn)*	FWD: ACCTGTCCCAGGTGTGTGAT REV: GCTGTGGATGATGTTGATGA
G6pc *(G6pc)*	FWD: CTACCTTGCGGCTCACTTTC REV: ATCCAAGTGCGAAACCAAAC
Glucokinase *(Gck)*	FWD: GATACCTGGGGAACAGCAAA REV: TAGGTGGAGACCCTGCTGAT
FOXO1 *(FOXO1)*	FWD:TCAGGCTAGGAGTTAGTGAGCA REV: GGGGTGAAGGGCATCTTT
FOXO3A *(FOXO3A)*	FWD:TTCAAGGATAAGGGCGACAG REV: GGCTGTGCAGTGACAGGTT
Mlx *(Mlx)*	FWD: TCTGTCCCCAACACAGATGA REV: ACGATGGCTTTGCTGAGTTT
MondoA *(Mlxip)*	FWD: ATCCACAGCGGCCACTTCATG REV: TCATGCACTCGAAGAGCTTGG
GFAT1 *(GFAT1)*	FWD: GCAGCCAGAATCTGTTGTGA REV: GCCATACCAGCGTGGTAACT
GFAT2 *(GFAT2)*	FWD: CCAGGCTGAAGAGACTGGAC REV: TCAGCCACTGCAGCAATATC
Pck1 *(Pck1)*	FWD: TGGCTACGTCCCTAAGGAA REV: GGTCCTCCAGATACTTGTCGA
PFKFB3 *(Pfkfb3)*	FWD: CACGGCGAGAATGAGTACAA REV: TTCAGCTGACTGGTCCACAC
L-PK *(Pklr)*	FWD: CTGGAACACCTCTGCCTTCTG

	REV: CACAATTTCCACCTCCGACTC
PTG *(Ppp1r3c)*	FWD: GAGGATTTGCTTGGCACATT REV: CTTGGAGTCAGCAAACACGA
GAPDH *(GAPDH)*	FWD: AGGGCTGCCTTCTCTTGTGAC REV: TGGGTAGAATCATACTGGAACATGTAG
CREB *(CREB)*	FWD: TTCTACAATATGCACAGACCACTG REV: CATCACCAGAGGCAGCTTG
TXNIP *(Txnip)*	FWD: ACCAGTGTCTGCCAAAAAGG REV: GCCATTGGCAAGGTAAGTGT
OGT *(OGT)*	FWD: ACCCTTCTACCCTGCAGATG REV: GCATATTGTTGAATATTGGGCTCT

2.1.7 Primers for ChIP assays

Primers were designed using gene sequence information from NCBI databases and the NCBI primer designing tool. Primers were synthesised by MWG Operon Biotech Ltd (Milton Keynes, UK) (table 2-6).

	Primers
G6pc (ChoRE region)	FWD: GCATCAGCCCTGTGTGAATA REV: GAGTTGAGGGCAAACAGAGC
G6pc coding region	FWD: CTACCTTGCGGCTCACTTTC REV: ATCCAAGTGCGAAACCAAAC
TXNIP (ChoRE region)	FWD: CGCACCCGAACAACAACCAT REV: AAGCGGGAGCCGGAAACGG
TXNIP coding region	FWD: ACCAGTGTCTGCCAAAAAGG REV: GCCATTGGCAAGGTAAGTGT

2.2 Hepatocyte isolation and culture

Rat hepatocytes were isolated by a two-step collagenase perfusion technique based upon the method of Seglen (Seglen, PO. 1976). The rat was killed by placing in a chamber saturated with Halothane vapour for 5 minutes. Briefly, after lapaotomy, the portal vein and superior vena cava were cannulated and the liver was perfused with Ca^{2+}-free EGTA-containing buffer (148 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 10 µg/ml phenol red, pH 7.4) at 20-30 ml/min for 15 min, followed by Ca^{2+} containing collagenase buffer (100 ml) (20

mg/100 ml collagenase, 124 mM NaCl, 6.7 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES, 10 μg/ml phenol red, pH 7.4) until digestion (15-20 min). The liver was then dissociated in MEM supplemented with 75 mg/l penicillin and 50 mg/l streptomycin, 2 mM glutamine and 0.1 mM non-essential amino acids. The cell suspension was filtered through 80 μm nylon mesh and centrifuged at 50 g to pellet viable cells. The cell pellet was washed 3 times and suspended in MEM supplemented with 5% (v/v) neonatal calf serum, non-essential amino acids, 2 mM L-glutamine, 75 mg/l penicillin and 50 mg/l streptomycin. Cells were seeded into gelatine coated (0.1% gelatine) multiwell plates and cultured at 37 °C equilibrated at 5% CO_2 and 95% air. After cell attachment (4 h) MEM was removed and replaced with fresh MEM containing 5 mM glucose with 10 nM dexamethasone and 10 nM insulin depending on the study, for overnight culture. For enzyme overexpression using recombinant adenoviruses, MEM was removed after attachment (2 h) and replaced by serum-free MEM containing various titres of adenovirus (2 h-4 h). The medium was removed and replaced with fresh MEM containing 10 nM dexamethasone and 10 nM insulin for overnight culture. Short-term experiments were carried out the following day (day1).

2.3 Preparation of Radioactive substrates for metabolic studies

For radiochemical assays, required amounts of [U-^{14}C] glucose (1 μCi/ml), D-glucosamine-1-^{14}C (4-5 μCi/ml), N-acetylglucosamine-^{14}C (2-3 μCi/ml), [2-^{3}H]glucose (1 μCi/ml), ^{3}H-leucine (0.4-0.5 μCi/ml) were dried down under nitrogen gas to remove the 90% ethanol, solvent. The dried isotope was then transferred to the MEM. A 50 μl aliquot of the radioactive MEM was mixed with

1ml scintillation cocktail and counted using a liquid scintillation analyser for determination of specific activity (TRI-CARB 2700).

2.3.1 Determination of glycogen synthesis

Glycogen synthesis was determined from the incorporation of [U-^{14}C] glucose into cellular glycogen which was isolated by ethanol (66%) precipitation. After overnight culture or short incubation with treatment, the cells were incubated with MEM containing [U-^{14}C] glucose and other additions as indicated for 3 hours. At the end of the incubation the medium was removed and cells were washed once with 0.15 M NaCl and then extracted with 0.1 M NaOH (0.4 ml / well) and frozen until later analysis. To precipitate protein, 250 µl of cell extract was added to 300 µl of 20% trichloroacetic acid (TCA) containing 0.75 mg/ml glycogen and the mixtures vortexed and finally centrifuged at 13,000 g, 4 °C for 15 minutes. 500 µl of supernatant was added to 1ml of 95% ethanol and centrifuged for 20 minutes at 13,000 g, 4 °C. The supernatant was then aspirated and the pellet washed twice with 1ml of 66% ethanol and centrifuged for 15 minutes at 13,000 g, 4 °C. After the second wash, the pellet was left to dry overnight.

The following day, 150 µl of deionised water was added to the pellet and heated at 55 °C for 15 minutes to dissolve the pellet. 1ml of scintillation cocktail was added to the samples and vortexed until clear. Radioactivity was then measured and results expressed as nmol of glucose incorporated into glycogen per 3 h per mg of protein.

Calculation

$$\text{Specific actvity} = \frac{\text{dpm}/\mu\text{l}}{\text{nmol glucose}/\mu\text{l}}$$

$$= \text{dpm/nmol}$$

$$\frac{\text{dpm}}{\text{SA}} \times \frac{\text{Total vol of cell ext. in well}}{\text{vol. added to glycogen/TCA}} \times \frac{\text{Total vol. of cell ext. + glycogen /TCA}}{\text{vol. added to ethanol}} \times \frac{1}{\text{Protein}}$$

(dpm/nmol)
(mg/well)

= nmol of glucose incorporated into glycogen per mg of protein.

2.3.2 Determination of incorporation of radiolabelled substrate into protein

Inorporation of ^{14}C-labelled glucose, ^{14}C- or ^{3}H -labelled glucosamine and ^{3}H-labelled leucine into protein was measured as described by (Simkin, JL. and Jamieson, JC. 1967).

Following overnight culture or short incubation with treatment, cells were incubated with MEM containing glucosamine-1-^{14}C (4-5 μCi/ml) or [U-^{14}C] glucose (1-2 μCi/ml) or ^{3}H-leucine (0.4-0.5 μCi/ml) or N-actylglucosamine-^{14}C (2-3 μCi/ml) in MEM for the time interval indicated. At the end of the incubation the medium was removed and cells were washed twice with 0.15 M NaCl. Cells were then extracted with 200 μl of extraction medium (containing 5 mg/ml of BSA, 20mM GlcN in 0.1 M NaOH) and sonicated. Protein was then precipitated by addition of 200 μl of 10% trichloroacetic acid (TCA) to 200 μl of cell extract followed by vortexing and centrifuged at 13,000 g, 4 °C for 15 minutes. The supernatant was aspirated and the pellet re-suspended in 500 μl of 5% trichloroacetic acid (TCA) containing 10mM GlcN followed by centrifugation for 10 minutes at 13,000 g, 4 °C. The following washes were then performed: (i)

5% TCA; (ii) 0.1 M NaCl/acetone (2:8); (iii) Ethanol/ether/chloroform (2:2:1); (iv) Ether (x2)

Following the final wash, pellets were left to air dry overnight. The following day, pellets were re-suspended in 150 µl of 0.1M NaOH and heated at 55 °C for 30 minutes. Radioactivity was then measured and results expressed as nmol of glucose incorporated into glycogen per 3 h per mg of protein.

Calculation

$$\text{Specific actvity} = \frac{\text{dpm/µl}}{\text{nmol glucose/µl}}$$

$$= \text{dpm/nmol}$$

$$= \frac{\text{dpm}}{\text{SA}} \times \frac{\text{Total vol of cell ext. in well}}{\text{vol. added to glycogen/TCA}} \times \frac{1}{\text{Protein}}$$

(dpm/nmol)
(mg/well)

= nmol of glucose incorporated into protein per 3h per mg of protein.

2.3.3 Determination of glucose phosphorylation

Glucose phosphorylation was measured from detritiation of [2-^{3}H] glucose (1-2 µCi/ml). After incubation, the medium was collected. Duplicate wells were pooled and 1 M HCl was added in the ratio of 10 % acid to the total sample volume. 50 µl of acidified sample was transferred to a 500 µl microcentrifuge tube which is placed inside a 5 ml scintillation vial containing 750 µl H_2O and stoppered. For blank correction, the tubes were incubated at 37 °C for at least 2 days to allow the ^3H_2O to equilibrate between the two solutions. To determine the level of tritiated water the small ependorf was removed and scintillation cocktail was added to the scintillation vial and counted. Results expressed as nmol of glucose detritiated per 3 h per mg of protein.

Calculation

$$\text{Specific actvity} = \frac{\text{dpm}/\mu\text{l}}{\text{nmol glucose}/\mu\text{l}}$$

$$= \text{dpm/nmol}$$

$$= \frac{\text{dpm samples} - \text{dpm blank}}{\text{SA (dpm/nmol)}} \times \frac{\text{Vol. of medium in well}}{\text{Vol. taken for assay}} \times \frac{\text{Total vol. of duplicate wells} + 1\text{M HCl}}{\text{Total vol. of duplicate wells}} \times \frac{1}{\text{Recovery}} \times \frac{1}{\text{Protein (mg/well)}}$$

= nmol of glucose detritiated per 3h per mg cell protein.

$$\text{Recovery factor} = \frac{^3H_2O \text{ in ependorf}}{^3H_2O + 750\mu\text{l } H_2O \text{ (total recovery)}}$$

2.4 Metabolite determination

2.4.1 Glucose 6-phosphate (glucose 6-P)

Glucose 6-P was determined using an endpoint fluorimeter coupled enzyme assay (Zhu et al. 2009). This method is based on the oxidation of glucose 6-P by glucose 6-P dehydrogenase (G6PDH) to yield NADPH, which is then used to reduce resazurin in the presence of diaphorase to produce resorufin (see principle of reaction below). Resorufin is highly fluorescent and can be detected by excitation at 530 nm and emission at 590 nm using a fluorimeter.

Principle of the reaction:

$$\text{Glucose 6-P} + \text{NADP} \xrightarrow{\textit{G6PDH}} \text{6-phosphogluconate} + \text{NADPH}$$

$$\text{NADPH} + \text{Resazurin} \xrightarrow{\textit{Diaphorase}} \text{NADP} + \text{Resorufin (Ex 530, Em 590)}$$

After incubation, cells were snap frozen in liquid nitrogen and plates stored at -80 °C, until extraction. Cells were extracted in 150 µl of 3% perchlorinc acid, sonicated, transferred to microcentrifuge tube and centrifuged for 10 minutes, 13000g at 4°C.

The supernatants (100 µl) were neutralised with 5 M KOH. The perchloric acid solution was first titrated against 5 M KOH using universal indicator to determine the exact volume for neutralisation. The acid extracts were then neutralised with the estimated volume of 3 M KOH without indicator which inteferes with the assay. Samples were cooled for a few minutes on ice to precipitate $KClO_4$ and centrifuged for 5 minutes, 13000g at 4°C.

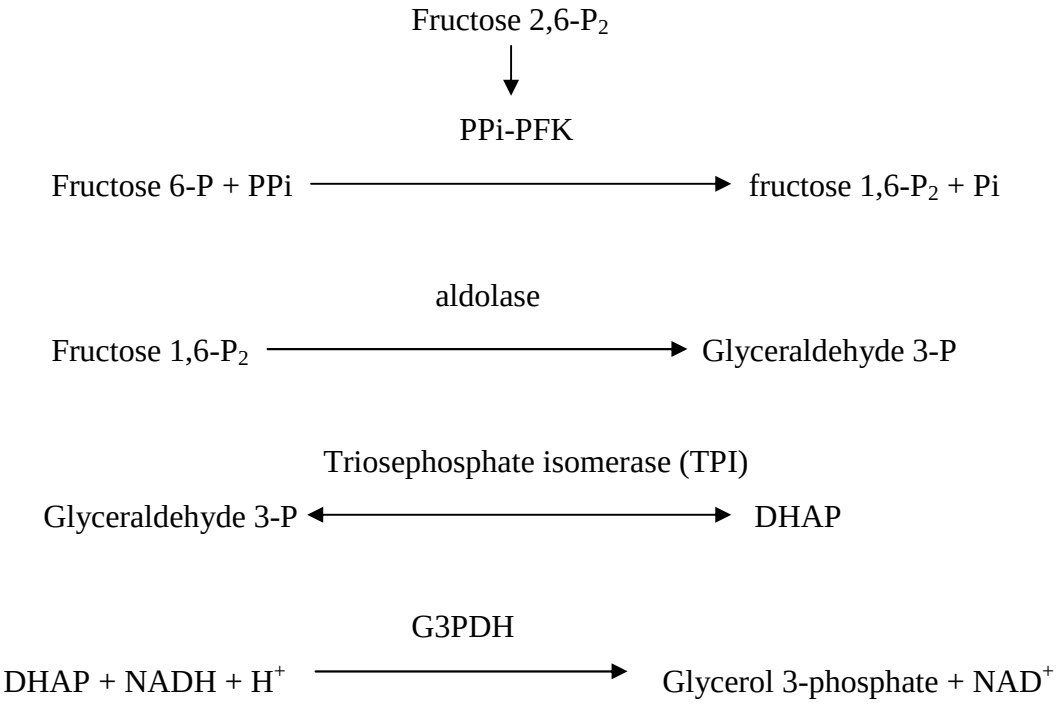
Glucose 6-P was then assayed using glucose 6-phosphate dehydrogenase from the rate of formation of NADPH. The assay cocktail contained 50 mM Tris-base neutralised with acetic acid to pH 7.8, 1 mM NADP, 2 mM EDTA and 0.03 Units / ml (5 µl of 1:5 dilution 700 U/ml) glucose 6-phosphate dehydrogenase.

Glucose 6-phosphate standards (5, 10 and 20 µM) were prepared in perchloric acid and neutralised similarly to the samples.

2.4.2 Fructose 2,6-bisphosphate ($F2,6-P_2$)

$F2,6-P_2$ was determined by measurement of NADH absorbance using kinetic analysis at 340 nm, 37°C. Decreased NADH is indicative of activation of PPi-PFK by $F2,6-P_2$ and is a measure of $F2,6-P_2$ concentration,:

Principle of the reaction:



On termination of incubations, the MEM was aspirated and 0.15 M NaOH was added (400 µl/well, 12-well plate), before the plate was heated at 80°C for 5 min. Plates were then cooled and stored at -20°C. On the day of the assay, standards of 5, 10, 20, 40, 60, 100, 150 and 300 nM F2,6-P_2 were prepared. Plates were thawed and samples were sonicated in the plate before 10 µl of samples and standards were added to a 96-well plate. 120 µl of main reagent (described below) was then added to each well and NADH absorbance was measured by kinetic analysis at 340 nm, 37°C. Levels of F2,6-P_2 were determined using a F2,6-P_2 standard curve and results are expressed as pmol/mg cell protein.

Main reagent: 0.1 M Tris-acetate, 2 mM Mg-acetate, 1 mM fructose 6-P* (described below), 0.15 mM NADH, Aldolase A2714 (0.5 U/ml), Glycerophosphate dehydrogenase / triosephosphate isomerase (1 U/ml), PPi-PFK (0.01 U/ml) and 0.45 mM pyrophosphate.

***Fructose 6-P was acidified to destroy contaminating F2,6-P_2:** 304 mg disodium salt was dissolved in 5 ml water. 2 ml of 1 mol/l HCl was then added and incubated at 37°C for 10 min, before the solution was neutralised with 0.95 ml of 2 M NaOH and aliquoted.

2.4.3 Determination of Glucosamine 6-phosphate(GlcN 6-P) and N-Acetylglucosamine (NAG)

Principle:

A modified colorimetric method for the estimation of GlcN 6-P and NAG was used (Morgan and Elson, 1934; Ghosh et. al., 1960).

Stock Reagents:

Ehrlich's reagent (0.5 g of 4-dimethylaminobenzaldehyde added to 625 µl of 10 M HCl and then diluted to 50 ml with glacial acetic acid) .

$NaHCO_3$, saturated solution

5% acetic anhydride solution (prepared fresh and kept cold)

0.8 M Sodium borate solution (pH 9)

5% (v/v) Perchloric acid (PCA) (4 ml 60% stock made up to 48 ml with water).

3 M K_2CO_3

Hepatocyte extraction and neutralisation:

After incubation, cells were snap frozen in liquid nitrogen and plates stored at -80 °C, until extraction. Cells were extracted in 5% perchloric acid (PCA) (150-200 µl for 6 well plates), transferred to ependorfs and centrifuged for 15 minutes, 13000g at 4°C.

The supernatants (100-150 µl) were neutralised with (16-24 µl) 3 M K_2CO_3 using universal indicator. Samples were cooled a few minutes on ice to precipitate $KClO_4$ and centrifuged for 5 minutes, 13000g at 4°C.

Standard preparation:

GlcN and NAG standards (5, 20, 50, 100, 150 and 200µM) were prepared in perchloric acid and neutralised similarly to the samples.

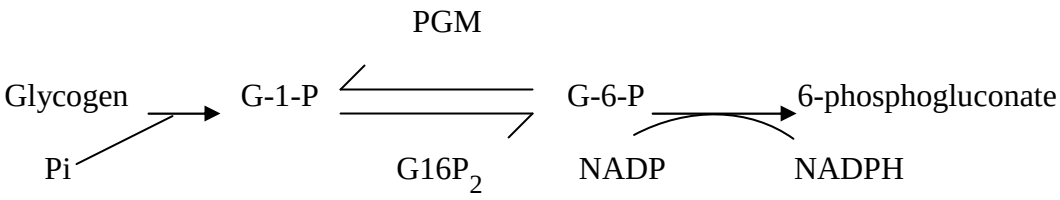
The Procedure:

80 µl of hepatocyte neutralised extracts and standards were treated with 10 µl of saturated $NaHCO_3$ solution and with or without 10 µl of ice-cold 5% acetic anhydride solution for determination of GlcN 6-P or NAG respectively. After vigorous shaking, the tubes were left at room temperature for 3 minutes and then heated in a heater block at 100 °C for 3 minutes to destroy excess acetic anhydride and the tubes cooled at room temperature. The tubes were treated with 20 µl of 0.8 M sodium borate solution (pH 9), mixed and then heated in a heater block at 100 °C for 3 minutes. After cooling, the tubes were treated with 600 µl of Ehrlich's reagent. The tubes were incubated for 20 minutes at 37 °C. The optical densities were measured in a spectrophotometer at 585 nm.

2.5 Enzyme activity determination

2.5.1 Determination of Glycogen phosphorylase-a (GPa)

Glycogen phosphorylase-a (GPa) activity was measured in the glycogenolytic direction spectrometrically by coupling to phosphoglucomutase (PGM) and glucose 6-phosphate dehydrogenase (G6PDH) and measuring NADPH production from the increase in absorbance at 340 nm.



After overnight culture or short incubation with treatment, cells were immediately snap frozen in liquid nitrogen and stored at -80 °C until use. 200 µl equivalent of extraction buffer (25 mM Hepes pH 7.5, 150 mM KF, 0.5 mM EDTA, 1 mM PMSF and 1 mM benzamidine) was added to 1 well of a 24 well plate, cells sonicated and centrifuged at 13,000 g, 4 °C for 6 minutes. Assays were performed in 96-well plates containing 50 µl of the sample supernatant and 200 µl of main reagent (50 mM Imidazole pH 7.5, 50 mM NaF, 0.5 mM EDTA, 2 mM $MgCl_2$, 0.5 mM Caffeine, 2 mg/ml glycogen, 0.5% of 100 mM NADP, 0.5% of 1 mM $G16P_2$, 0.33 U/ml PGM and 0.44 U/ml G6PDH, 2.5 mM phosphates. Enzyme activity was monitored for 18 minutes and determined from the linear rate of increase in absorbance at 340 nm. Enzyme activities are expressed as milliunits per mg cell protein, where 1 munit is the amount of enzyme needed to convert 1 nmol of substrate per min.

2.5.2 Determination of GFAT activity

Glutamine:fructose 6-phosphate amidotransferase (GFAT) activity was determined from an end point assay with glutamine and fructose 6-P to form GlcN 6-P, which was then measured after acetylation by a modified colorimetric method for the estimation of GlcN 6-P and NAG (Ghosh, S. et. al., 1960).

Hepatocytes were extracted in 200 µl of buffer containing 25 mM HEPES, 150 mM KF, 0.5 mM EDTA, 1 mM PMSF, 1 mM benzamidine (pH 7.5), 2mM

glutamine, 2mM fructose 6-phosphate and protease inhibitor cocktail (1/1000). Cells were transferred to microcentrifuge tube and incubated for 1 hour at 37 °C and then heated in a heater block at 100 °C for 3 minutes to destroy the enzyme. Levels of GlcN 6-P and NAG were determined as described in section 2.4.3.

2.6 Determination of mRNA and protein expression

2.6.1 Semi-quantitative real-time (RT)-PCR

This study utilised semi-quantitative real-time (RT)-PCR for determination of steady state mRNA levels. RNA was extracted from rat hepatocyte monolayers using Trizol reagent. Culture medium was removed and cells washed once with 1 x saline (150 mM NaCl) followed by the addition of 250 µl trizol (24-well plates) and vigorous scraping. Trizol was then transferred to individual eppendorfs and incubated at room temperature. After 5 minutes 200 µl of chloroform was added and samples shaken vigorously for 15 seconds followed by incubation at room temperature for 2 minutes. Samples were then spun at 12,000g, 4˚C for 15 minutes and the aqueous upper phase transferred to fresh eppendorf tubes and isopropanol (0.5 ml per 1ml TRIZOL extracted) added and mixed thoroughly. Following incubation at room temperature for 10 minutes, samples were spun at 12,000g, (4˚C, 10 minutes) and supernatant removed. Pellets were washed with 250 µl 75% ethanol (diluted in pure H_2O, Sigma-Aldrich, UK), and re-pelleted by a final centrifugation at 7,500g, 4˚C for 5 minutes. Ethanol was aspirated and samples air dried at room temperature for 10 minutes. Finally, pellets were resuspended in 10 µl H_2O and incubated at 55˚C for 10 minutes.

Reverse Transcription

Genomic DNA was removed by treatment with RNase free DNase I for 15 minutes at 37°C, followed by denaturation of the enzyme at 70°C for 10 minutes. The RNA was quantified on Spectromax (260:280 nm). Primer random (1 μg/ml) was added to 1 μg RNA and samples incubated for 10 minutes at 70˚C. Samples were incubated for 1 hour containing 1 x RT buffer, 10mM DTT, 250 μM dNTP mix, 5 U/μl Superscript II reverse transcriptase at 42˚C or M-MLV reverse transcriptase at 37˚C. The total volume of cDNA was 40 μl and the final concentration was 25ng/μl. RT-PCR was performed with 50ng of reverse transcribed RNA, in a final volume of 10μl PCR reaction mixture containing 0.5μM of forward and reverse primer, 3mM $MgCl_2$ and 1 x lightCycler FastStart DNA Master SYBR. There are four programmes designed for PCR; pre-incubation and denaturation, amplification, melting curve and cooling. Samples were denatured at 95˚C for 10 minutes, followed by 40 cycles consisting of 95˚C for 15 seconds, 58˚C for 7 seconds and 72˚C for 15 seconds.

2.6.2 Western blotting

Hepatocytes were extracted in a buffer containing 25 mM HEPES, 150 mM KF, 0.5 mM EDTA, 1 mM PMSF, 1 mM benzamidine (pH 7.5), followed by sonication. The samples were diluted with 0.25 volumes of 4 x SDS loading buffer containing 0.5 M Tris, 10 % SDS (w/v), 10 % glycerol, 4 % mercaptoethanol and 0.1 % bromophenol blue in ethanol and denatured at 100 °C for 5 min. The samples were loaded into SDS polyacrylamide gel for electrophoresis (180 V for 60 min). After electrophoresis, the gels were electrophoretically transferred (semi-dry) onto nitrocellulose membranes (ECL

Hybond N, Amersham Biosciences Buckinghamshire, UK) using a Trans-Blott SD semi-dry transfer cell from Biorad Laboratories Ltd. (Hertfordshire, UK) at 15 V for 50 min. 50 ml transfer buffer (30 g Tris base, 144 g glycine, 150 ml methanol and 800 ml water) was used. Membranes were stained with Ponceau Red (0.1 %) to verify equal amounts of protein between lanes prior to Western blot analysis. After Ponceau Red staining the membranes were blocked 1h in TBST (3.035 g Tris base, 8.4 g NaCl, 2 ml concentrated HCl, 5 ml Tween-20 and 1 liter water) containing 5 % BSA. Membranes were incubated with the primary antibody overnight. Membranes were washed vigorously four times in TBST. They were then incubated with the peroxidase conjugated anti-rabbit/mouse IgG secondary antibody for 1h. They were washed four times in TBST as before. Membranes were developed using ECL chemiluminescence reagent according to the manufacturer's instructions followed by brief exposure to ECL X-ray film.

2.6.3 Determination of cellular protein

Cellular protein on the NaOH (0.1M) extracts was measured spectrometrically by a modified Lowry method. The basic principle of this reaction is that an active constituent of Folin-phenol reagent, phosphomolybdic-tungstic acid, is reduced by protein to form a coloured complex which can be measured at 750 nm.

A combination of start and main reagents are used in the assay. The main reagent (10% (w/v) Na_2CO_3 in 0.5 M NaOH, H_2O, $CuSO_4.5H_2O$ and Na^+/K^+ tartate in a 10:40:1:1 ratio) is activated with Folin and Ciocalteu's phenol diluted with distilled water in a 1:1 (v/v) ratio. BSA standards at 0.25, 0.5 and 1 mg/ml were diluted in 0.1M NaOH and used for the standard curve.

2.7 Immunoprecipitation assays

2.7.1 Wheat germ agglutinin (WGA) precipitation

Hepatocytes were extracted in a buffer (pH 7.5) containing 25 mM HEPES, 150 mM KF, 0.5 mM EDTA, 1 mM PMSF, 1 mM benzamidine and 1:1000 with protease inhibitor cocktail, followed by addition of 30ul WGA agarose beads with or without 5mM N-acetylglucoseamine (NAG) for 2h at 4 °C. After three washes with extraction buffer with or without 5mM NAG, the samples were diluted 1:1 with 4 x SDS loading buffer containing 0.5 M Tris, 10 % SDS (w/v), 10 % glycerol, 4 % mercaptoethanol and 0.1 % bromophenol blue in ethanol and denatured at 100 °C for 5 min. The samples were then processed by western blotting as detailed in section 2.6.2.

2.7.2 Chromatin Immunoprecipitation (ChIP)

2.7.2.1 Chromatin Immunoprecipitation

ChIP assays were performed using the ChIP Assay Kit protocol (Millipore) with slight modifications. Cross-linking of hepatocytes was performed by addition of formaldehyde to incubation medium to a final concentration of 1% for 10 min at 37 ºC, before addition of glycine to a final concentration of 0.125M for 5 min at room temperature to quench the cross-linking reaction. Samples were washed twice and harvested with ice cold PBS containing 1mM PMSF and 1:300 protease inhibitor cocktail. Cells were pelleted by centrifugation for 4 min at 2,000 g and resuspended in 3ml SDS lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris, pH

8.1). Cell lysates were then sonicated using Bioruptor UCD-300 on high setting with 10 cycles of 30 seconds on and 20 seconds off, to give sheared DNA fragments between 100 and 1000bp in length, then centrifuged at 13,000g for 10 min at 4 ºC and 500µl cell supernatants were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl). Diluted cell supernatants were pre-cleared with 75µl protein A agarose / salmon sperm DNA (50% slurry) containing 600µg sonicated salmon sperm DNA (Sigma-Aldrich cat no. 31149), 1.5mg BSA and 4.5mg recombinant protein A agarose (Upstate Biotechnology cat no. 16-125) suspended in TE buffer, pH 8.0. Following centrifugation at 1,000g for 2 min to pellet agarose, cell supernatants were incubated with 6µg of antibodies against either IgG, Acetyl-H4, ChREBP, MondoA, FOXO1, FOXO3A or NF-Y, overnight with rotation at 4 ºC. IgG, Acetyl-H4, ChREBP, MondoA, FOXO1, FOXO3A and NF-Y immunocomplexes were collected by incubation with 120µl protein A agarose/salmon sperm DNA (50% slurry), whilst Mlx immunocomplexes were collected by incubation with 120µl protein G agarose (Upstate Biotechnology cat no. 16-266) / salmon sperm DNA (50% slurry) for 3 h with rotation at 4 ºC. Beads were washed with low salt (catalog no. 20-154), high salt (cat. no. 20-155), LiCl (cat. no. 20-156) and TE (cat. no. 20-157) wash buffers for 5 min each with rotation at 4 ºC. Immune complexes were eluted by addition of 250µl elution buffer (1% SDS, 0.1M $NaHCO_3$) for 30 min with rotation at room temperature. Elution was performed twice and the eluates combined to give a total volume of 500µl, and cross-links were reversed with addition of 20µl 5M NaCl and heating at 65 ºC for 4 hr. Samples were then incubated with 0.5M EDTA, 1M Tris-HCl, pH 6.5 and Proteinase K for 1 h at 45ºC. DNA was recovered by phenol/chloroform extraction.

2.7.2.2 Phenol/chloroform extraction

Phenol/chloroform extraction was performed on eluted immune complexes (approximately 500µl total volume). 500µl phenol/chloroform/isoamyl alcohol mixture was added to eluted immune complexes and samples vigorously shaken before being centrifuged at 13,000g for 5 min. 450µl of supernatant was transferred to fresh eppendorf tubes and combined with 950µl 95% ethanol and 25µl 10M NH_4OAc before centrifugation at 13,000g for 5 min. Supernatants were removed using a needle and syringe and pellets were washed with 250µl 70% ethanol, before samples were vortexed and centrifuged at 13,000g for 2 min. Ethanol was removed using a needle and syringe and pellets were left to air dry for approximately 15 min. Pellets were then resuspended in 10µl molecular biology grade H_2O and heated at 55ºC for 10 min.

2.7.2.3 Touchdown real-time PCR

Touchdown real-time PCR was performed on DNA recovered by phenol/chloroform extraction using 1µl DNA, 0.5µmol/l of each primer (**listed in Table 2.6**) and 1x LightCycler FastStart DNA Master SYBR Green I in a final reaction volume of 10µl in LightCycler capillaries run on a Roche LightCycler. Samples were denatured at 95°C for 10 min followed by 2 cycles of 95°C for 15 sec, 68°C for 7 sec (annealing) and 72°C for 15 sec (elongation). The annealing temperature was then lowered by 1°C every 2 cycles until the final annealing temperature of 58°C was reached, whereby 30 cycles of 95°C for 15 sec, 58°C for 7 sec and 72°C for 15 sec were performed. This method of real-time PCR gives a

selective advantage to the specific PCR product over the spurious non-specific products that can appear at lower annealing temperatures, and thereby improves the sensitivity and specificity of the PCR reaction. Melt curve analysis was also performed to confirm amplification of specific transcripts only, and all the primer sets listed in **Table 2.6** produced only a single peak on their respective melt curves, therefore confirming specificity of all primer sets used.

2.8 Immunostaining

Immunostaining was performed to determine the changes in subcellular localisation of ChREBP, FOXO1, FOXO3A and SREBP1c in response to different substrates in hepatocytes. Hepatocytes were cultured on glass coverslips that had been sterilised with ethanol, flame dried and placed in 24-well plates.

2.8.1 Fixation

Upon termination of short-term incubations, MEM was aspirated and coverslips were washed twice with PBS. Cells were fixed by addition of 4% paraformaldehyde (described below) and incubation for 30 min at room temperature. Paraformaldehyde was then aspirated and coverslips were washed twice with 1xPBS.

4% paraformaldehyde: 1g paraformaldehyde was dissolved in 50ml nanopure H_2O (heated to 70-80°C) with 200µl 2M NaOH and 5ml 10xPBS. Paraformaldehyde was then cooled on ice before being used for fixation.

2.8.2 Immunofluorescence

500µl 1mg/ml sodium borohydride (made up in 1xPBS) was added to each coverslip and incubated for 10 min at room temperature to quench autofluorescence of hepatocytes. Coverslips were then washed twice with 1xPBS and 500µl 0.2% Triton-X100 (made up in PBS) was added and incubated for 10 min at room temperature to permeabilise the cells and improve antibody penetration. Coverslips were then washed twice with 1xPBS and transferred to a 12-well plate lined with parafilm. 25µl 1% BSA / 0.2% Triton-X100 was added to each coverslip and incubated for 10 min at room temperature to act as a blocking solution to reduce background and unspecific staining. Cells were then washed twice with 1xPBS and 25µl primary ChREBP, FOXO1, FOXO3A or SREBP1c antibodies (described below) were added to each coverslip and incubated for 2 – 3 h at room temperature or overnight at 4°C. Primary antibody was removed and cells washed with 1xPBS three times for 10 min each on a shaker, before 25µl Alexa Fluor 488-conjugated anti-rabbit or mouse secondary antibody (described below) was added to each coverslip and incubated for 1 h at room temperature. Secondary antibody was then removed and cells were washed with 1xPBS three times for 10 min each on a shaker. 0.2µg/ml DAPI was then added to cells and incubated for 5 min at room temperature to stain nuclei before cells were washed twice with 1xPBS, then placed on a paper towel and allowed to air dry for 20 – 30 min.

Primary antibody (1:150 dilution) (volume per coverslip)

0.17µl ChREBP, FOXO1, FOXO3A or SREBP1c antibodies

2.5µl 10% BSA in PBS

12.5µl 0.2% Triton-X100 in PBS

9.83µl 1xPBS

Secondary Alexa Fluor 488-conjugated antibody (1:50 dilution) (volume per coverslip)

0.5µl Alexa Fluor 488-conjugated antibody

2.5µl 10% BSA in PBS

12.5µl 0.2% Triton-X100 in PBS

9.5µl 1x PBS

2.8.3 Mounting and imaging of coverslips

Once coverslips had air dried, they were mounted face down onto glass slides using Mowiol R-4088 containing 2.5% DABCO to help prevent fading of the immunofluorescent dyes (Valnes and Brandtzaeg 1985). Coverslips were left to dry and then examined using a Nikon E400 fluorescent microscope. Images were taken at 40x magnification and captured using a Nikon DXM1200 digital camera. For quantification, >100 cells were imaged for each substrate condition and ChREBP, FOXO1, FOXO3A or SREBP1c localisation was scored as to whether: i) cytoplasm > nuclear; ii) nuclear ≥ cytoplasm. The percentage of cells displaying either greater cytoplasmic or nuclear ChREBP, FOXO1, FOXO3A or SREBP1c was then calculated.

2.9 Statistical analysis

Results are expressed either as means ± SEM or are representative of the number of experiments indicated. Statistical analysis was performed with the Student's paired t-test experiments. A P value of < 0.05 was considered to be statistically significant.

Chapter 3

The Hexosamine pathway: flux measurements and concentrations of pathway intermediates

The Hexosamine pathway: flux measurements and concentrations of pathway intermediates

3.1 Aims and rationale

After glucose enters the liver cell it is metabolised to glucose 6-phosphate by glucokinase which is a key regulatory step in glucose metabolism. Glucose 6-phosphate is either metabolised by glycogenesis or the pentose phosphate pathway or it is converted to fructose 6-P which can either enter into glycolysis or the HBP. According to Marshall and colleagues from studies on adipocytes and other cell types, about 2-5 % of total intracellular glucose enters the HBP (Marshall et al., 1991). The HBP involves the conversion of fructose 6-P and glutamine to GlcN 6-P, a reaction catalysed by the rate-limiting step enzyme glutamine:fructose 6-phosphate amidotransferase (GFAT). GlcN can also enter the HBP by phosphorylation to GlcN 6-P which bypasses the rate-limiting GFAT step. Conversion of GlcN to GlcN 6-P is catalysed by glucokinase (Van Schaftingen, 1995). GlcN 6-P is ultimately converted into uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) which is the substrate for various glycosylation modifications which include N-GlcNAcylation and O-GlcNAcylation of proteins in the ER and Golgi compartments or O-GlcNAcylation of protein in the cytoplasm or nucleus (Copeland et al., 2008; Hart et al., 2011) (Fig. 3.1).

One of the enzymes that uses UDP-N-acetylglucosamine as substrate is O-linked beta-N-acetylglucosamine transferase (OGT) which catalyses the transfer of O-GlcNAc to serine and threonine residues (Copeland et al., 2008; Wang et al., 2009). This reaction is reversed by the enzyme beta-N-acetylglucosaminidase (OGA or also known as O-GlcNAcase) which catalyses the removal of the O-GlcNAc group. The role of O-linked glycosylation of proteins has been studied

using inhibitors of OGT and OGA or by overexpression of these proteins. Various studies have suggested a role for this covalent modification in the regulation of insulin action by regulating Akt on Ser^{473} and Thr^{303} in hepatoma cells (HepG2) (Soesanto et al., 2008) and in adipose tissue (Yang et al., 2008). Studies on adipose tissue have reported that the HBP plays an important role in regulating gene expression of the lipogenic enzyme FAS and ACC (Rumberger et al., 2003). A recent study in hepatocytes reported that the HBP plays a key role in insulin resistance by inducing gene expression of G6Pc (Dentin et al., 2008). Other studies have reported that overexpression of GFAT which increases flux through the HBP results in ER stress and accumulation of lipid by induction gene expression of fatty acid synthase (FAS) in hepatoma cells (HepG2) (Sage et al., 2010).

The aims of this study were: first, to test the validity of GFAT inhibitors and substrates that enter the HBP after the GFAT catalysed reaction as potential tools to study the role of the HBP in control of gene expression; second, to determine whether the hepatocellular concentrations of down-stream intermediates of the HBP are altered by various substrates that enter the HBP either before or after GFAT; third, to estimate flux through the HBP in hepatocytes at glucose concentrations that are commonly used to study glucose-regulated gene expression; fourth, to test whether inhibitors of OGT and OGA can be used as potential tools to study the role of covalent modification of proteins in cell regulation.

3.2 Results

3.2.1 Glutamine: fructose-6-phosphate amidotransferase (GFAT) activity in hepatocytes and effects of inhibitors

Previous studies have used azaserine (AZN) and 6-diazo-5-oxo-l-norleucine (DON) at concentrations of 20-40 μM to inhibit GFAT activity and flux through the HBP in adipocytes (Marshall et al., 1991). These studies showed that these inhibitors block the insulin resistance caused by high glucose in various non-hepatic cells (Zachara and Hart, 2006; McClain and Crook, 1996). However, the effects of these inhibitors on GFAT activity in hepatocytes have not been reported. The first aim was to determine the concentrations of AZN and DON and incubation time required to inhibit GFAT activity in primary hepatocytes. Hepatocytes were incubated with AZN or DON at 20 μM for either 4 h or 18 h and the enzyme activity of GFAT was determined in supernatants of hepatocyte lysates by incubation with fructose 6-P and glutamine in an end-point assay 1h (Ye et al., 2003). On termination of the assay the product (GlcN 6-P) was acetylated and determined by the method of Morgan and Elson (Morgan and Elson, 1934; Ghosh et al., 1960). The assay was validated from sample blanks from which glutamine was omitted. In contrast with previous studies on adipocytes where AZN was shown to be a potent inhibitor of GFAT at concentrations of 2-10 μM in 18 h incubation (Marshall et al., 1991), in this study there was only 17% inhibition of GFAT activity after 18 h incubation with 20 μM AZN (Fig 3.2, A). However, there was 70% inhibition with 20 μM DON after 18 h. Figure 3.2 B, shows concentration-dependent inhibition of GFAT activity by DON (5, 10, 20 and 40 μM) on GFAT activity after 18 h (39, 68, 73 and 96 %). The results show maximum inhibition at 40 μM DON.

3.2.2 GFAT mRNA expression in hepatocytes and absence of effect of DON

The activity of GFAT in rat liver has different kinetic properties from that in other tissues (Huynh, QK. et al. 2000). There are two isoforms of GFAT encoded by separate genes: GFAT1, which is expressed in all tissues and GFAT2 which differs in its kinetic and regulatory properties and is expressed in liver (Ye et al., 2004; Huynh et al., 2000; Zhou et al., 1998; Oki et al., 1999; Hu et al., 2004). The next aim was to determine gene expression of GFAT (GFAT1 and GFAT2) with or without 40 µM DON. Hepatocytes were incubated with the GFAT inhibitor DON at 10, 20 and 40 µM for 18 h and mRNA levels were determined. The results showed that there was no effect of DON on GFAT 1 and GFAT2 mRNA expression (Fig. 3.3).

3.2.3 Absence of effect of DON on glucose phosphorylation

After finding that GFAT activity in hepatocytes can be inhibited by more than 90% by 40 µM DON during culture for 18 h we next tested whether this treatment affects glucose phosphorylation, the first reaction in glucose metabolism. This was measured using [2-^{3}H] glucose which measures the glucokinase catalysed reaction. There was no significant effect of 20 µM and 40 µM DON on glucose phosphorylation (Fig 3.4). This suggests that DON (40 µM for 18 h) can be used to inhibit GFAT without inhibiting glucose phosphorylation.

3.2.4 Measurement of intermediates of the hexosamine biosynthesis pathway: effects of substrates

3.2.4.1 Effects of glucose and GFAT inhibitors on accumulation of NAG-metabolites in hepatocytes

Having established the concentration of DON and incubation time required to inhibit GFAT activity in hepatocytes, the next aim was to determine the effects of glucose and other substrates on the concentrations of metabolic intermediates of the HBP and also to determine whether DON affects the concentrations of these intermediates. In the HBP, GlcN 6-P the first intermediate is converted by acetylation to N-acetylglucosamine (NAG 6-P) which is metabolised to N-acetylglucosamine 1-P (NAG-1-P) and then to UDP-N-acetylglucosamine. NAG-metabolites were measured by the method of Morgan and Elson which measures all N-acetylglucosamine metabolites. Incubation of hepatocytes with 25 mM or 50 mM glucose for 4 h caused a 2-3 fold increase in concentration of NAG-metabolites relative to 5 mM glucose (Fig 3.5). Pre-treatment of the hepatocytes with 20 μM DON for 18 h partially counteracted the increase in NAG-metabolites caused by high glucose. While pre-treatment with 40 μM DON totally counteracted the increase in NAG-metabolites caused by 25 mM and 50 mM glucose (Fig 3.5).

3.2.4.2 Effect of glutamine and octanoate on NAG-metabolites in hepatocytes

We tested the effects of glutamine which is a substrate for GFAT (Fig 3.1) and octanoate which is a source of acetyl units for acetylation of GlcN 6-P to N-acetylglucosamine 6-P (Fig 3.6, A,B) on the total concentration of NAG-metabolites in hepatocytes incubated with 5 mM or 25 mM glucose for 4 h. These experiments showed that 10mM glutamine caused a small but significant increase in concentration of NAG-metabolites at high glucose (Fig. 3.6A) and octanoate also caused a significant increase NAG-metabolites at high glucose (Fig. 3.6B).

3.2.4.3 Effects of glucosamine (GlcN) and N-acetylglucosamine (NAG) on N-Acetylglucosamine metabolites

The above studies show that accumulation of NAG metabolites of the HBP is increased between 2 and 3 fold by 25 mM and 50 mM glucose (Fig. 3.5). The next aim was to compare the effects on the accumulation of NAG metabolites of: (i) GlcN, which enters the HBP after GFAT and is generally used at concentrations as high as 10 mM in studies of the HBP in hepatocytes and hepatoma cells (Dentin et al., 2008; Taylor et al., 2009) and (ii) NAG which enters the HBP after the acetylaton of GlcN 6-P. The effects of a range of concentrations of GlcN and NAG on the accumulation of HBP intermediates (NAG-metabolites) were tested. These experiments showed that GlcN (0.5 to 10 mM) caused a concentration dependent accumulation of HBP-metabolites with a similar effect of 0.5mM GlcN as high glucose concentration. However, higher concentrations of GlcN (1, 5 and 10mM) caused a much larger stimulation (9-50-fold) than glucose (Fig. 3.7, A). NAG (10 mM and 20 mM) also caused a

significant increase in NAG-metabolites but this was much smaller than the increase caused by GlcN (Fig 3.7, B). This could be because NAG is transported into hepatocytes less efficiently than GlcN (Van Schaftigen, 1995) or because it is phosphorylated by a different kinase from GlcN.

3.2.4.4 Accumulation of GlcN 6-P with GlcN

Because glucosamine caused a large accumulation of NAG-metabolites (Fig. 3.7, A) in further experiments the effect of GlcN on both GlcN 6-P and NAG metabolites was determined by using a modification of the method of Morgan and Elson (Morgan and Elson, 1934; Ghosh et al., 1960), involving acetylation of the samples with acetic anhydride to convert GlcN 6-P to NAG 6-P. The acetylated sample measures the sum total of GlcN 6-P and NAG-metabolites and the difference between acetylated and non acetylated samples (NAG-metabolites) measures GlcN 6-P. From this data we can see that at 1 mM and 5 mM GlcN there is accumulation of both GlcN 6-P and NAG-metabolites, but at 10 mM GlcN there is a much greater accumulation of GlcN 6-P than of NAG-metabolites (Fig 3.8). This means that above 5 mM GlcN, the enzyme glucosamine 6-phosphate N-acetyltransferase (GlcN 6-P NATase) becomes the rate limiting enzyme resulting in accumulation of its substrate.

3.2.4.5 Effects of GlcN and NAG on glucose phosphorylation, glucose 6-phosphate and phosphorylase-a in hepatocytes.

The above experiments demonstrate that both GlcN and NAG cause a large increase in NAG metabolites accumulation. The next step was to determine the

effects of GlcN on glucose phosphorylation and glucose metabolism, to test whether GlcN concentrations can be found that raise NAG-metabolites without interfering with other metabolic pathways of glucose metabolism. The initial experiments tested the effects of a range of concentrations of GlcN (0.1 to 5.0 mM) on glucose phosphorylation, glucose 6-P, glycogen synthesis and the activity of glycogen phosphorylase. These experiments showed that GlcN caused a concentration dependent inhibition of glucose phosphorylation (Fig. 3.9A), lowering of glucose 6-P (Fig. 3.9B), activation of glycogen phosphorylase-a (Fig. 3.9C) and inhibition of glycogen synthesis (Fig. 3.9D), with significant effect at 0.5 mM GlcN for glucose phosphorylation, glucose 6-P and glycogen synthesis ($P < 0.05$) and 2.0 mM GlcN for glycogen phosphorylase-a ($P < 0.05$).

Because GlcN is a competitive inhibitor of glucokinase, the inhibition of glucose phosphorylation by GlcN is probably explained by a direct effect of GlcN on glucokinase. This was tested in hepatocytes overexpressing glucokinase (Fig. 3.9E). In these experiments which have a higher glucokinase activity, as shown by the 2-fold increase in glucose phosphorylation (Fig. 3.9E), significant inhibition occurred at higher GlcN concentration (2 mM rather than 0.5 mM). In cells expressing endogenous glucokinase (Fig. 3.9A-D) GlcN inhibited glucose phosphorylation at the same concentrations (0.5 mM) that causes an increase in NAG metabolites (Fig. 3.7). This means that GlcN cannot be used to selectively increase HBP flux without inhibiting glucose phosphorylation and other pathways of glucose metabolism. We therefore tested the effects of NAG, which causes a smaller increase in NAG-metabolites than GlcN (Fig.3.7B). However, 10 mM NAG also caused a significant inhibition of glucose phosphorylation similar or greater than the inhibition by 0.5 mM GlcN (Fig. 3.9F).

3.2.4.6 Mechanism of activation of phosphorylase-a by GlcN

The inhibition by both GlcN and NAG of glucose phosphorylation could be caused by inhibition of glucokinase because both compounds are strong glucokinase inhibitors (Vandercammen and Van Schaftingen, 1991) or by an independent mechanism. We therefore compared the effect of GlcN with a glucokinase inhibitor that is not an amine (5-thioglucose (5TG)) and with two amines that are not glucokinase inhibitors such as galactosamine (GalN) and methylamine (MethN). Surprisingly all compounds tested lowered glucose 6-P and raised phosphorylase a, but the increase in phosphorylase-a was greatest with GlcN (Fig. 3.10). When glucokinase was overexpressed it prevented the increase in phosphorylase-a caused by the glucokinase inhibitor, 5TG, but it did not prevent the activation of phosphorylase-a by GlcN (Fig. 3.11). Because glucokinase overexpression increased glucose 6-P to basal with 2 mM GlcN but it did not prevent the activation of phosphorylase, these results suggest that the effect of GlcN on phosphorylase is at least in part independent of the lowering of glucose 6-P. Possible mechanisms that could explain the activation of phosphorylase by GlcN that is not prevented by glucokinase overexpression are calcium release from the ER which would be expected to cause activation of phosphorylase kinase through calmodulin (Bollen et al., 1987).

3.2.5 Flux through the hexosamine pathway (HBP) measured with GFAT inhibitors

3.2.5.1 Validation of GFAT inhibitors for studying flux through the pathway at varying glucose concentration: effects on glucose phosphorylation and protein synthesis

The next aim was to determine the rate of the HBP in hepatocytes from the incorporation of [U-^{14}C] glucose into protein in comparison with the rate of glucose phosphorylation. The rate of glucose phosphorylation incubated with 25 mM glucose was 226 nmol / 4 h per mg protein and this was not significantly affected by DON (20-40 μM) as shown previously (Fig 3.4). Time course incubations (up to 4 h) of the incorporation of glucose into protein determined using [U-^{14}C]glucose showed that this was linear with time and also that addition of GlcN decreased the incorporation of ^{14}C-glucose into protein (Fig. 3.12A). The incorporation of ^{14}C-glucose into protein was then determined in 3h incubations in cells treated without or with cycloheximide (CX) to inhibit protein synthesis or 40 μM DON to inhibit GFAT (Fig. 3.12B. Protein synthesis was also determined from leucine incorporation into protein in parallel incubations (Fig. 3.12C). CX inhibited protein synthesis (leucine incorporation) by 90% and it inhibited glucose incorporation into protein by 70%. This shows that incorporation of glucose into protein (24 nmol / 4 h per mg protein) is in part due to labelling of amino acids and in part (~ 20%) to other mechanisms that are not due to protein synthesis.

In the presence of 40 μM DON the incorporation of glucose into protein was inhibited by 20% (from 24 to 18 h). If DON fully inhibits the HBP and has negligible effect on protein synthesis then the contribution of the HBP to ^{14}C-

glucose incorporation into protein is around 20%. The DON-sensitive rate of ^{14}C-glucose incorporation corresponded to 6 nmol/4 h per mg protein. This is approximately 3% of the rate of glucose phosphorylation (226 nmol/4 h per mg) measured in similar incubation conditions (Fig 3.4). These calculations assume that DON does not affect protein synthesis. In the leucine experiments there was a small though not significant effect of DON on protein synthesis. This suggests that DON may slightly overestimate the contribution of the HBP.

To further confirm that the incorporation of ^{14}C-glucose into protein represents the hexosamine pathway additional incubations were performed without or with NAG which is expected to dilute the incorporation of label from glucose into protein. At concentrations of 5 mM and 10 mM, NAG caused a greater inhibition of incorporation of ^{14}C-glucose into protein than for inhibition of glucose phosphorylation (Fig. 3.13). This is consistent with ^{14}C-glucose incorporation into protein by covalent modification through the HBP.

3.2.5.2 Validation of OGT and OGA inhibitors from protein labelling with ^{14}C-glucose, ^{14}C-glucosamine and ^{14}C-N-acetylglucosamine

Studies on covalent modification of proteins by OGT often use inhibitors of OGT or inhibitors of OGA to alter the covalent modification of proteins. We next used radioactive labelling of proteins with radioactive [U-^{14}C]glucose, glucosamine-1-^{14}C and N-acetylglucosamine-1-^{14}C to test whether inhibitors of OGT (benzyl-2acetamido-2-deoxy-alpha-D-galactopyranoside (BADGP), alloxan) and OGA (PUGNAc) alter total protein labelling in hepatocytes. Tunicamycin (TM), an inhibitor of N-linked glycosylation was used as a control. Hepatocytes were incubated with radioactive glucose, glucosamine and N-acetylglucosamine for 4 h

and protein was precipitated (after lipid extraction to eliminate incorporation of glucose, GlcN and NAG into lipid). These studies showed no effect of 5 mM alloxan but significant inhibition by BADGP (10 mM) of glucose, GlcN and NAG labelling and inhibition by tunicamycin of GlcN and NAG labelling (Fig. 3.14). Although BADGP caused significant inhibition it also caused some cell death. An effect of BADGP on cell stress was evident from the increase in phosphorylase (Fig. 3.15). When the effects of alloxan, BADGP and tunicamycin were tested in 4 h incubation with the radioactive labels (Fig. 3.16), both tunicamycin and BADGP inhibited GlcN incorporation into protein (similar to the inhibition of NAG incorporation) and they also inhibited protein synthesis (Fig. 3.16A, B). Unexpectedly PUGNAc an inhibitor of OGA which is expected to have opposite effects as BADGP on GlcN labelling had no effect (Fig. 3.16A). To test whether the inhibitory effects of BADGP on GlcN labelling may be due to non-specific effects we determined the incorporation of ^{14}C-glucose into glycogen, which is very sensitive to cell stress involving an increase in cytosolic calcium (Fig. 3.16C). In these experiments, BADGP but not tunicamycin inhibited label incorporation into glycogen. This is consistent with the activation of glycogen phosphorylase by BADGP (Fig. 3.15) and also with the changes in cell morphology. Together, these results do not provide support for use of either BADGP or PUGNAc as inhibitors of OGT and OGA that affect covalent modification of proteins without non-specific effects.

3.2.5.3 O-GlcNAc covalent modification of protein in hepatocytes

We used an antibody to O-GlcNAc to covalent modification of proteins glycosylation by high glucose, overexpression of OGT, GlcN and inhibitor of OGA (PUGNAc) in hepatocytes incubated with 5 mM or 25 mM glucose by immunoblotting. These experiments showed that 25 mM glucose increased O-GlcNAc modification to protein compared with 5mM glucose and both 0.5 mM GlcN and 100 uM PUGNAc also increased O-GlcNAc immunoactivity at 25 mM glucose (Fig. 3.17).

3.3 Discussion

Glucose metabolism by the HBP and modification of proteins via O-GlcNAc are both suggested to be involved in the development of insulin resistance. The role of the HBP has been studied from experiments with GFAT inhibitors and incubations with GlcN. The role of covalent modification of proteins with O-GlcNAc has been studied from experiments with GlcN which causes covalent modification of protein and from overexpression of OGT and OGA (Yang et al., 2008; Soesanto et al., 2008) and from experiments with inhibitors of these enzymes (Sakiyama et al., 2010; D'Alessandris et al., 2004). The majority of studies on the role of the HBP in insulin resistance were performed on adipocytes and muscle cells and other non-liver cell lines with relatively few studies on liver or hepatocytes (Dentin et al. 2008). Liver differs from other tissues in its activity and kinetic properties of GFAT which are at least partly due to expression of the GFAT2 isoform. To study the role of the HBP in control of gene expression in hepatocytes it was first necessary to establish valid concentrations of inhibitors and substrates that enter the HBP that are appropriate for liver cells. This study therefore tested the effects of commonly used GFAT inhibitors and substrates of the HBP in hepatocytes as well as inhibitors of OGT and OGA.

3.3.1 GFAT inhibitors

Azaserine (AZN) and 6-diazo-5-oxonorleucine (DON) are inhibitors of GFAT (Marshall et al., 1991). Previous studies on adipocytes reported that AZN was a potent inhibitor of GFAT at a concentration of 2-10 μM during 18 h incubation (Marshall et al. 1991). The present study found that the enzyme activity of GFAT

was only inhibited by 9% following incubation with 20 μM of AZN for 18 h, whilst with 20 μM of DON for 18 h the inhibition was 67%. The optimal concentration of DON was found to be 40 μM for an 18 h incubation, which completely blocked the enzyme activity of GFAT (96%). The weak inhibitory effect of azaserine in hepatocytes may be due to the different isoform of GFAT expressed in hepatocytes (Ye et al., 2004; Huynh et al., 2000; Zhou et al., 1998; Oki et al., 1999; Hu et al., 2004) or to other differences in amino acid metabolism in liver. The long incubation time required for inhibition of GFAT by DON is explained by covalent modification of the enzyme in the presence of DON (Sage et al., 2009; Marshall et al., 1991). Treatment of hepatocytes with 40 μM DON for 18 h had no significant effect on the rate of glucose phosphorylation as determined by the phosphorylation of [$^{2\text{-}3}$H]glucose which is the general method for measurement of glucokinase flux in hepatocytes. It also had little effect on the rate of protein synthesis in hepatocytes as determined from the incopororation of labelled leucine into protein. Further support for the validity of DON was obtained from the measurement of NAG metabolites which represent the intermediates of the HBP that are formed after the acetylation of GlcN 6-P. High glucose concentrations caused a moderate (2-3 fold) increase in the concentrations of NAG metabolites and this effect was prevented by treatment with 40 μM DON. These results together support the validity of DON as an experimental tool to determine the role of the HBP in hepatocytes incubated with high glucose concentration.

3.3.2 GlcN and NAG

Incubation with high concentrations of GlcN is the most commonly used method to show the importance of HBP in the development of insulin resistance (Sage et al., 2009; Dentin et al., 2008; Taylor et al., 2009). In addition a recent study that reported regulation of G6Pc gene expression through the HBP in hepatocytes used GlcN at a concentration of 10 mM (Dentin et al., 2008). In this study we compared the effects of GlcN with the effects of high glucose concentration on the accumulation of NAG metabolites. High glucose (25 mM to 50 mM) caused an increase in NAG metabolites of between 2-fold and 3-fold in comparison with basal glucose concentration (5 mM). This increase in NAG-metabolites is similar to the increase in glucose 6-P or the concentrations of intermediates of glycolysis between the fed and fasted states in the liver in vivo (Casazza and Veech, 1986). However, with millimolar concentrations of GlcN as are often used in studies on the HBP (Dentin et al., 2008), the increase in NAG metabolites was about 10-20 fold greater than with high glucose. In addition GlcN also caused accumulation in GlcN 6-P. This metabolite was not detectable in incubations without GlcN indicating that although it is an intermediate of the HBP it is normally present in the hepatocytes at very low concentrations. This could either be explained by a lower activity of GFAT compared with the activity of the glucosamine 6-phosphate N-acetyltransferase (GlcN 6-P NATase) the second enzyme in the HBP, or by feed-back inhibition of GFAT activity by GlcN 6-P, which would prevent accumulation of this metabolite from glucose. The increase in GlcN 6-P concentration was very high at 5 mM or 10 mM GlcN. This indicates that in these conditions the GlcN 6-P NATase becomes rate limiting. The high accumulation of this phosphorylated intermediate can cause depletion of cell ATP and also

inorganic phosphate. NAG which unlike GlcN is not a substrate for glucokinase but is phosphorylated by another kinase, N-acetylglucosamine kinase (Berger et al., 2002), caused a much smaller increase in NAG metabolites than GlcN. This lower rate of accumulation of NAG-metabolites can also be in part due to a slower rate of transport of this sugar analogue into hepatocytes. Unlike DON, which had no effect on glucose phosphorylation, both GlcN and NAG were found to inhibit glucose phosphorylation. Both compounds are inhibitors of glucokinase and the inhibition of glucose phosphorylation is probably best explained by direct inhibition of glucokinase. The lowering of glucose 6-P can be explained by the inhibition of glucose phosphorylation, and the inhibition of glycogen synthesis can be explained by the lowering of glucose 6-P, which is an allosteric activator of glycogen synthase. This conclusion is supported by the experiments with glucokinase overexpression. However, the activation of glycogen phosphorylase by GlcN can be caused by two mechanisms: the lowering of glucose 6-P and by calcium release from the ER which causes activation of phosphorylase kinase. The activation of phosphorylase was not prevented by overexpression of glucokinase. The lowest concentrations of GlcN and NAG (0.5 mM and 10 mM) that caused a significant increase in NAG metabolites also caused inhibition of glucose phosphorylation.

In summary, this study shows that GlcN and NAG are more effective than glucose at raising NAG-metabolites in hepatocytes. However, unlike DON they also cause inhibition of glucose phosphorylation. They therefore cannot be used to alter flux through the HBP without also affecting metabolism through other metabolic pathways.

3.3.3 Incorporation of labelled glucose, GlcN and NAG into protein

As expected from the entry of GlcN and NAG into the HBP after the rate limiting step, experiments comparing the incorporation of radiolabelled glucose, GlcN and NAG into protein showed much higher labelling of protein with GlcN and NAG compared with glucose. In addition, experiments using cycloheximide (CX) to inhibit protein synthesis showed that 70% of the glucose label that was incorporated into protein was due to labelling of amino acids that are used for protein synthesis. It was estimated from comparison of the effects of CX on label incorporation from with either glucose or leucine into protein that about 20% of the glucose labelling of protein was caused by covalent modification of protein. From the effects of DON on the incorporation of labelled glucose into protein it was also estimated that the contribution of the HBP to glucose labelling of proteins was about 20%. This calculation assumes a selective effect of DON on the HBP and no inhibition of protein synthesis. Although labelling of protein by leucine was not significantly decreased by DON, there was a trend towards lower incorporation. However, this was less than for glucose labelling inhibition by DON.

The experiments testing the effects of commonly used inhibitors of OGT and OGA did not identify inhibitors that are free of cytotoxic effects at concentrations that cause changes in covalent labelling of proteins. PUGNAc a commonly used inhibitor of OGA had no effect on label incorporation into protein. Experiments testing the effects of two commonly used OGT inhibitors on the covalent modification of protein with GlcN, NAG or glucose showed that BADGP but not alloxan inhibited labelling of protein. However, BADGP also caused inhibition of protein synthesis as measured from leucine incorporation and it also caused

activation of phosphorylase and inhibition of glycogen synthesis suggesting the induction of cell stress. Therefore the most commonly used inhibitors of OGT and OGA are not useful experimental tools to alter the covalent labelling of proteins with O-GlcNAc in hepatocytes.

3.4 Summary

This study has shown that:

- The GFAT inhibitor DON (40uM) inhibits GFAT activity in hepatocytes after 18 h culture by ~96% and has little or no effect on glucose phosphorylation, protein synthesis or GFAT mRNA expression. It can therefore be used as an experimental tool to study the HBP in hepaotyctes.

- The concentration of NAG-metabolites but not that of GlcN 6-P was significantly raised at 25mM glucose compared with 5mM glucose indicating that glucose is metabolised by the HBP at 25mM. This effect of glucose was inhibited by DON confirming its validity as a tool to the study the HBP.

- GlcN was a very effective substrate for the HBP and it caused a larger increase in NAG metabolites than glucose. At high concentrations GlcN also caused a large increase in GlcN 6-P indicating that GlcN 6-P NATase becomes rate limiting. However, GlcN is also a very strong inhibitor of glucose phosphorylation and therefore affects other pathways of glucose metabolism.

- NAG is a better substrate than glucose but a weaker substrate than GlcN at increasing NAG metabolites. Unlike GlcN it dos not increase GlcN 6-P but it is also a glucokinase inhibitor.

- Flux through the HBP was determined from the DON-sensitive incorporation of ^{14}C-glucose into protein and was 3% of glucose phosphorylation.

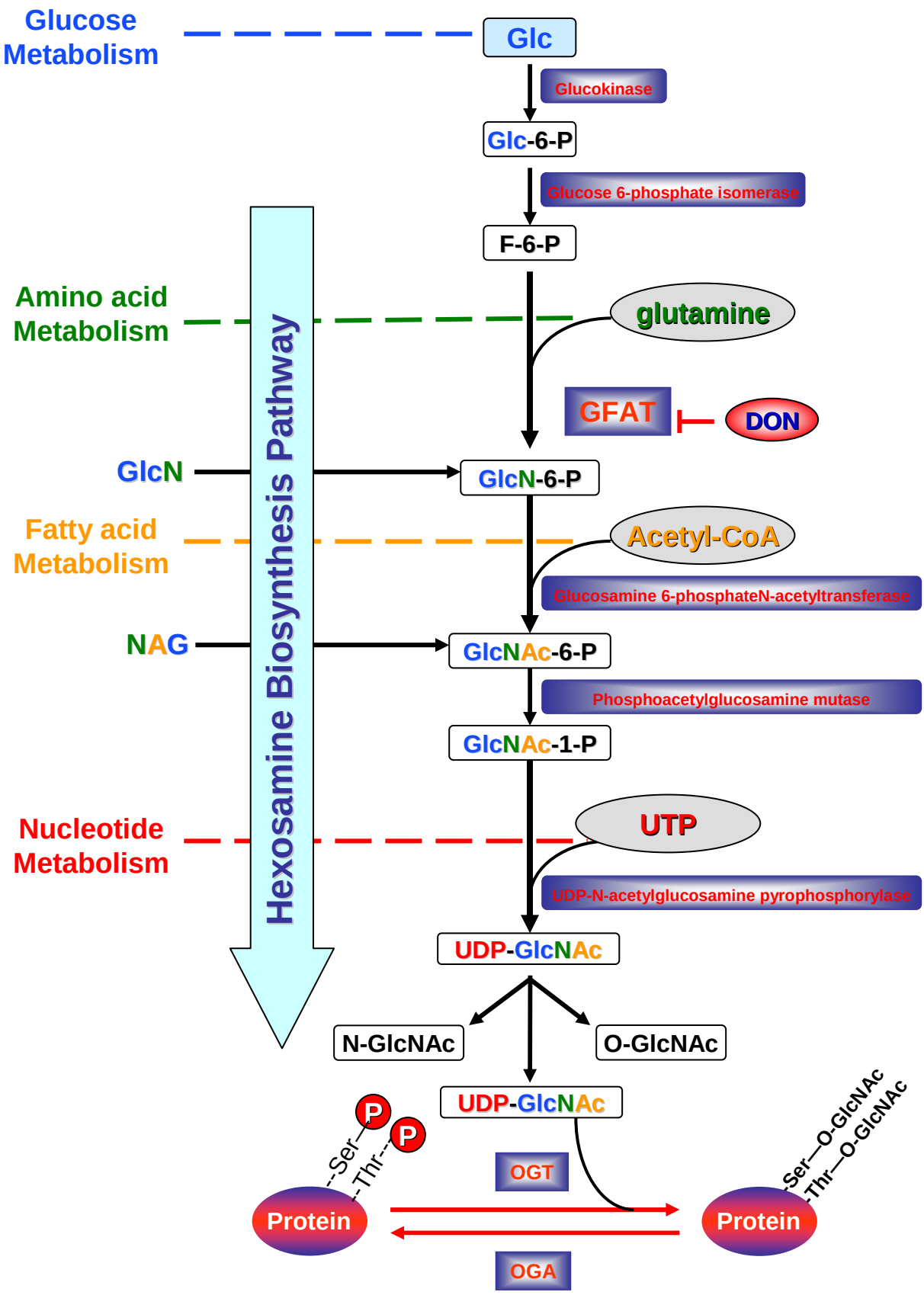


Figure 3.1 The hexosamine biosynthesis pathway and modification of proteins via O-GlcNAc (Hart et al., 2011).

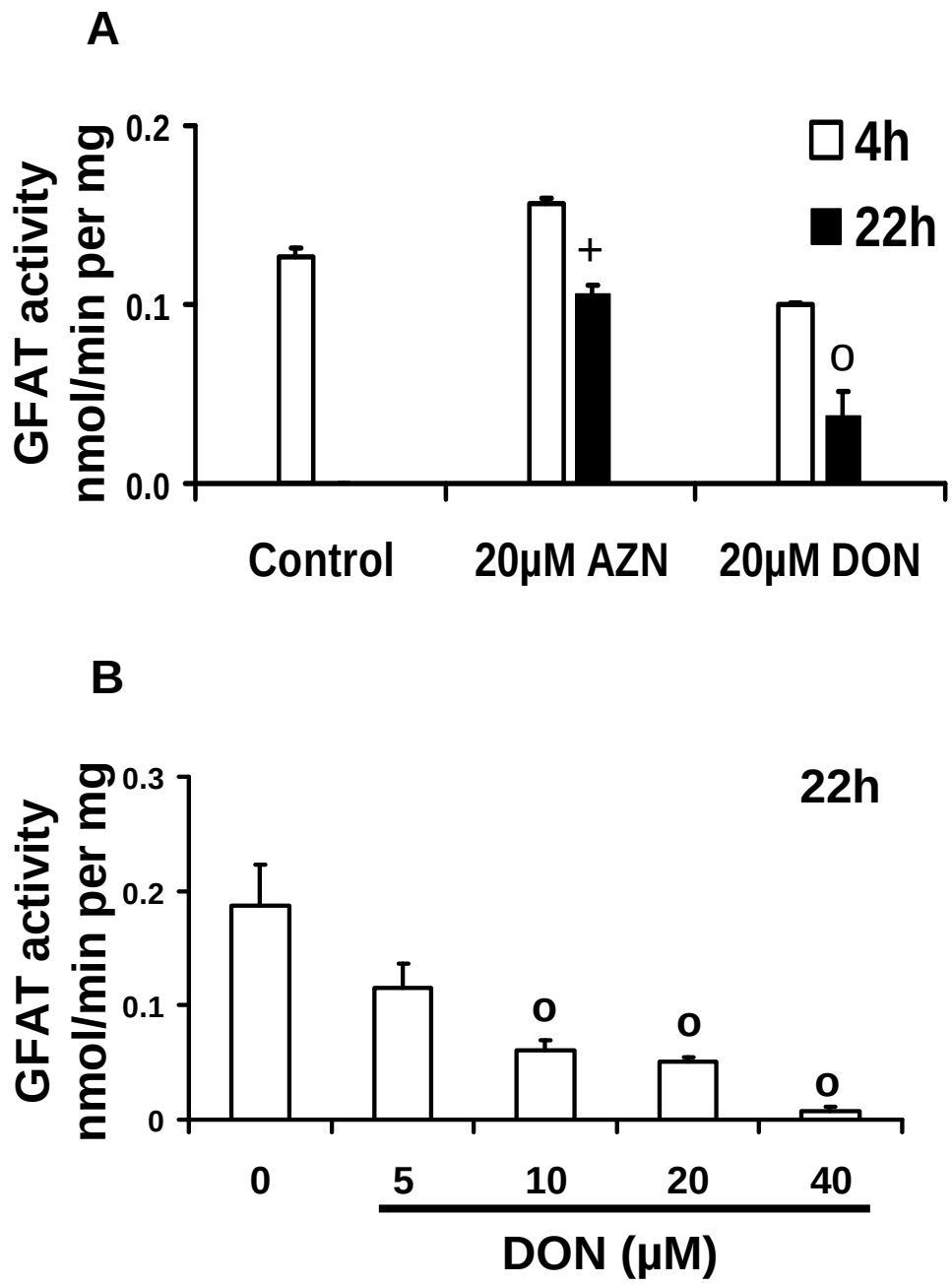


Figure 3.2 Effects of azaserine (AZN) and 6-diazo-5-oxonorleucine (DON) on glutamine:fructose-6-phosphate amidotransferase (GFAT) activity
Hepatocyte monolayers were pre-cultured 18 h in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- AZN and DON. They were then incubated for 4 h or 22 h in MEM containing 5 mM glucose, with 20 μM AZN and DON (**A**) and the concentrations of DON (**B**) indicated for determination of GFAT activity. Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6). ^{o} $P < 0.05$ effects of DON $^{+}$ $P < 0.05$ effects of AZN

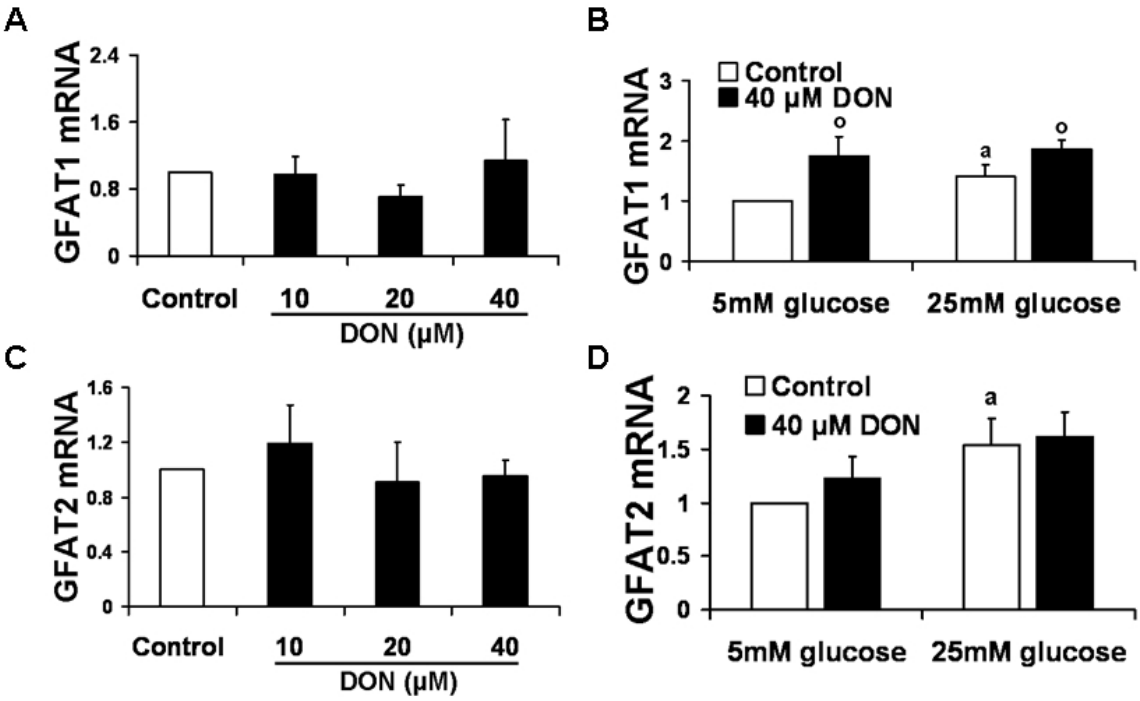


Figure 3.3 Effects of 6-diazo-5-oxonorleucine (DON) on gene expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 or 25 mM glucose, with 10, 20 and 40μM DON and indicated for determination of gene expression of (A,B)GFAT1, (C,D) GFAT2. Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12). , $^{a}P< 0.05$ effect of glucose, $^{o}P< 0.05$ effect of DON.

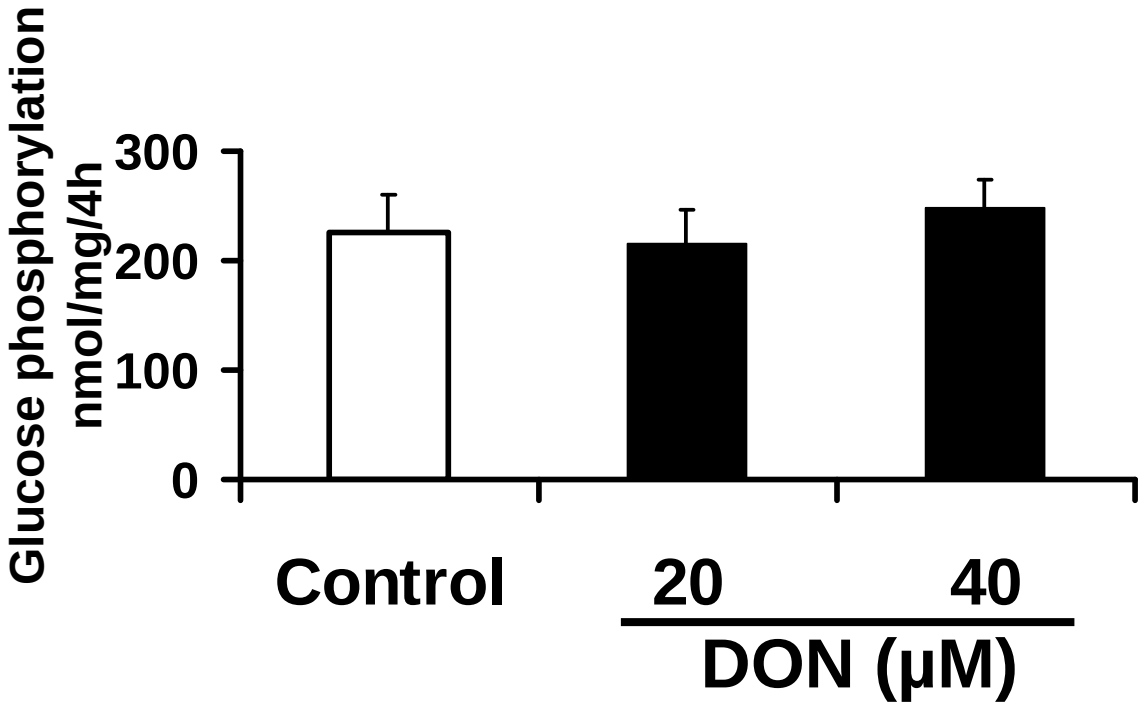


Figure 3.4 Effects of 6-diazo-5-oxonorleucine (DON) on glucose phosphorylation

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 25 mM glucose 2-^{3}H-glucose (3 uCi /ml) with 20 and 40 μM DON indicated for determination of glucose phosphorylation. Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6). .

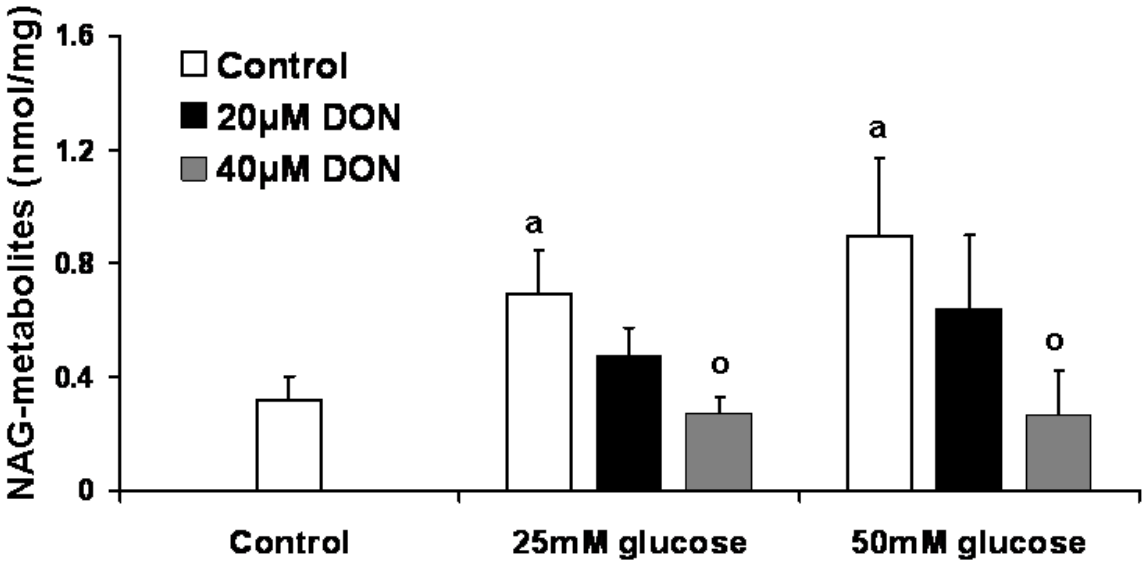


Figure 3.5 Effects of 6-diazo-5-oxonorleucine (DON) on N-acetylglucosamine (NAG) metabolites

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5, 25 and 50 mM glucose, with 20 and 40µM DON indicated for determination of N-acetylglucosamine metabolites. Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6). ^{a} $P < 0.05$, effects of glucose, ^{o} $P < 0.05$, effects of DON.

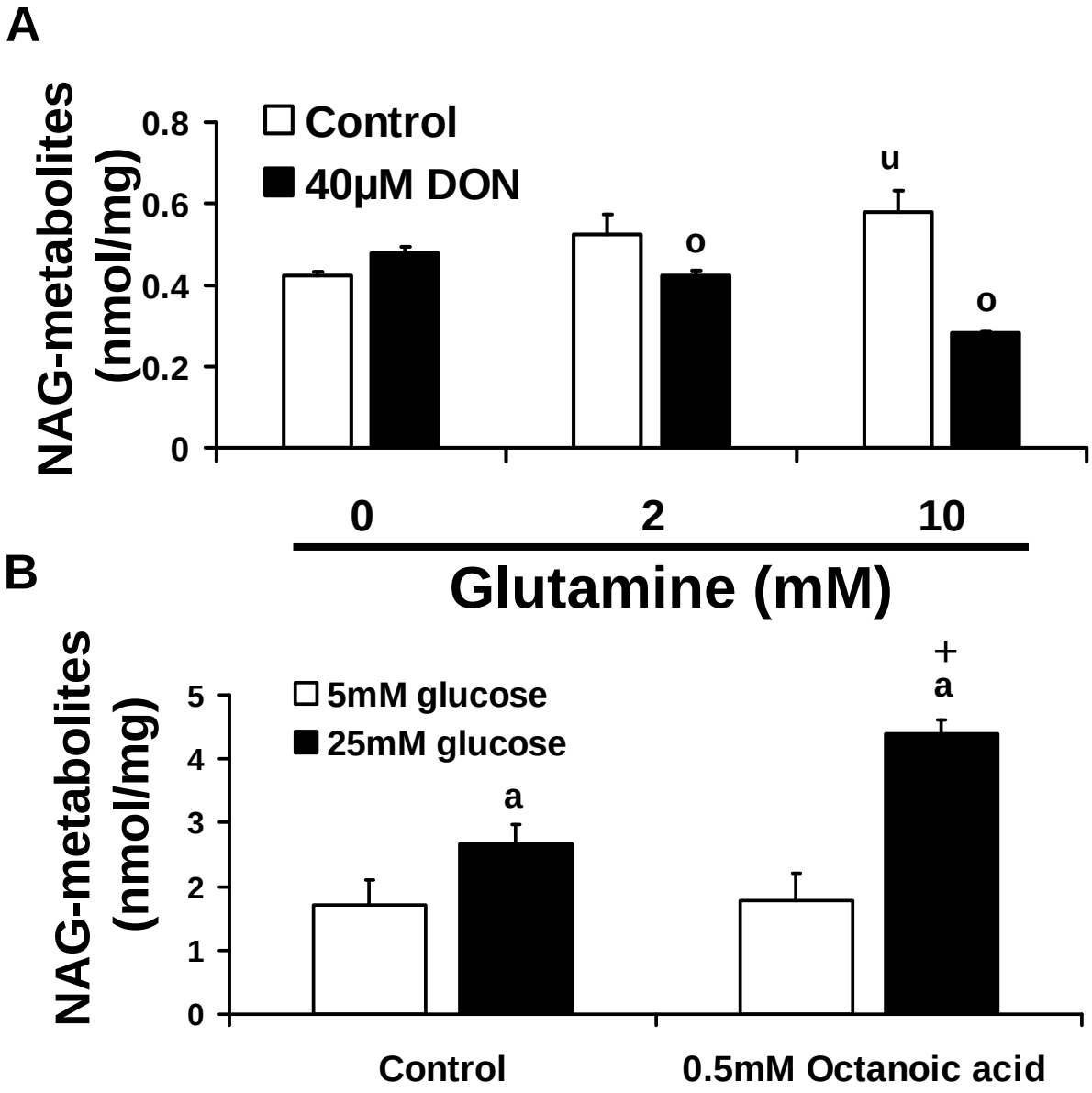


Figure 3.6 Effects of glutamine and octanoate on N-acetylglucosamine (NAG) metabolites

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 25 mM glucose with 0, 2 and 10 mM glutamine and 40 µM DON (**A**) indicated for determination of N-acetylglucosamine metabolites. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose with 0.5 mM octanoate (**B**) for determination of N-acetylglucosamine metabolites. Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6). ^{a} $P < 0.05$, effects of glucose, ^{o} $P < 0.05$ effects of DON, $^{u}P < 0.05$, effects of glutamine and $^{+}P < 0.05$, effects of octanoate

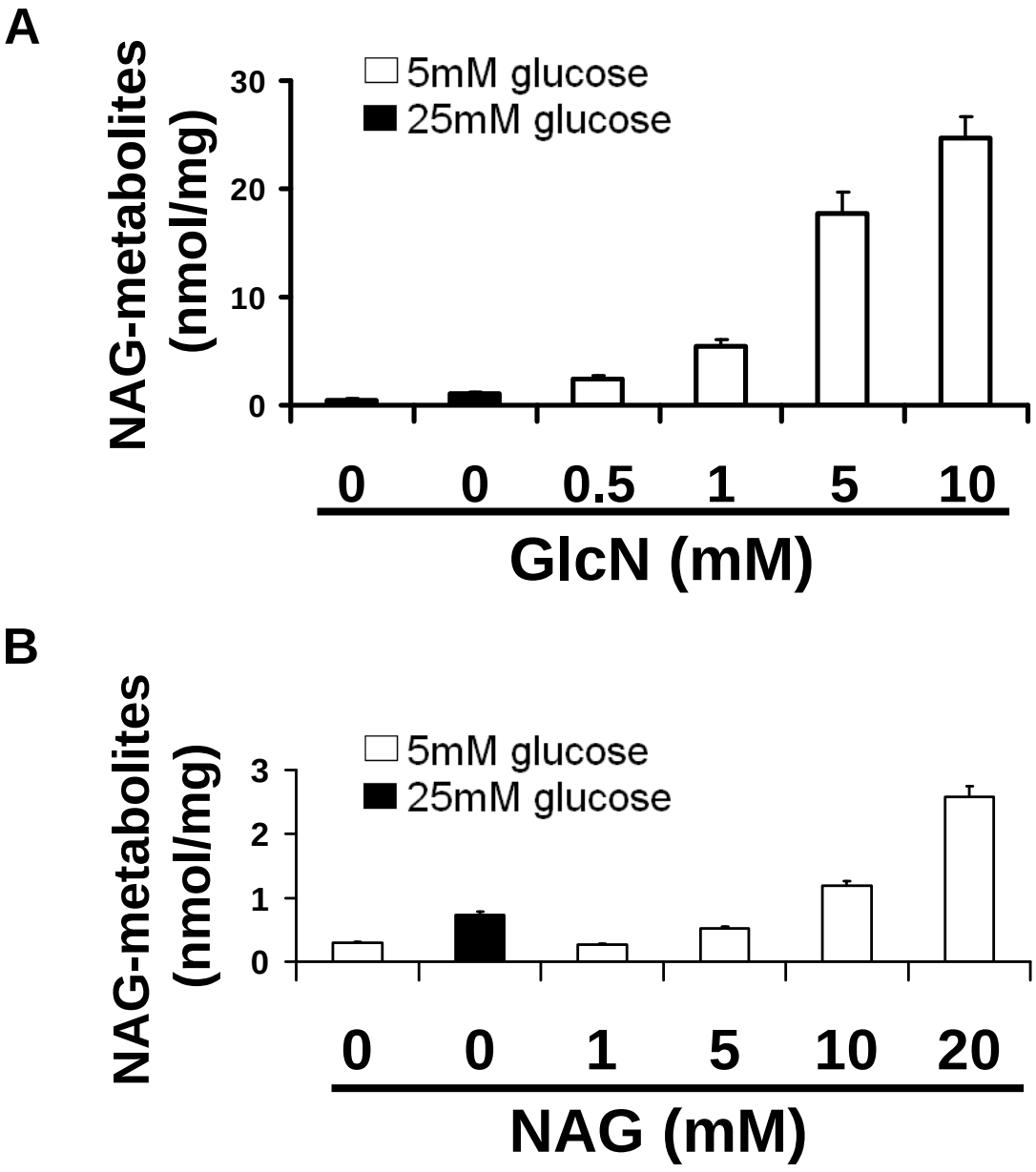


Figure 3.7 Effects of glucosamine (GlcN) and N-acetylglucosamine(NAG) on N-acetylglucosamine metabolites

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose, with 0.5, 1, 5 and 10 mM glucosamine (GlcN) (A) and 1, 5, 10 and 20 mM N-acetylglucosamine (NAG) (B), indicated for determination of N-acetylglucosamine metabolites. Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6).

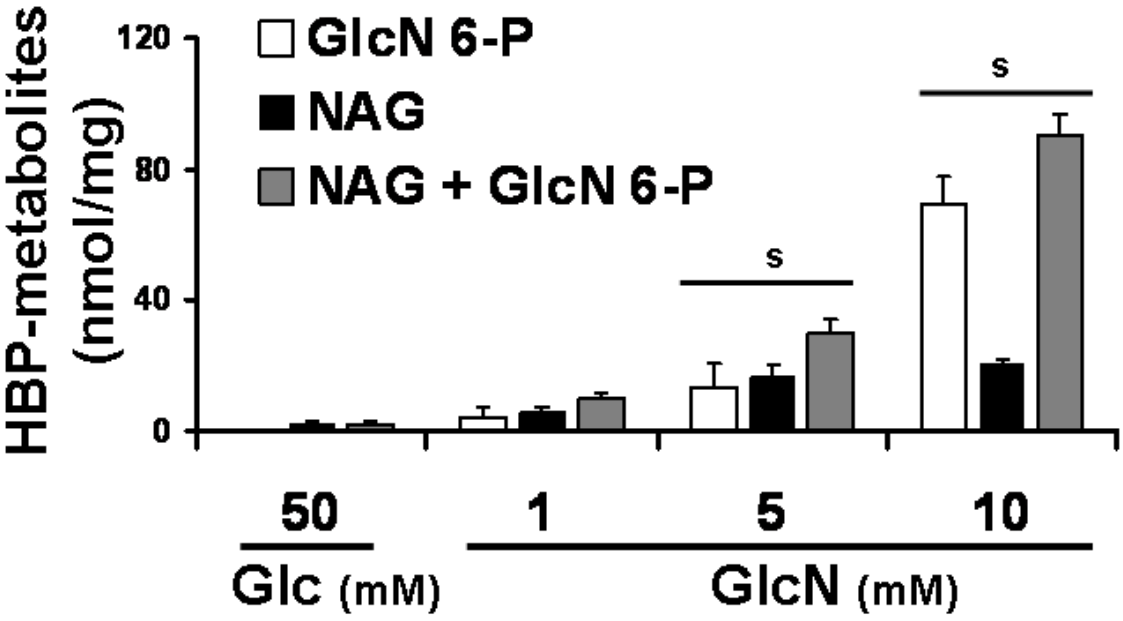


Figure 3.8 Effects of glucosamine concentration on GlcN 6-P and N-acetylglucosamine metabolites

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 50 mM glucose (Glc) and glucosamine (GlcN) (1, 5 and 10 mM), indicated for determination of GlcN 6-P, N-acetylglucosamine metabolites and both. Results are expressed as means ± SEM for 3 experiments. ^{S} $P < 0.05$ effects of glucosamine.

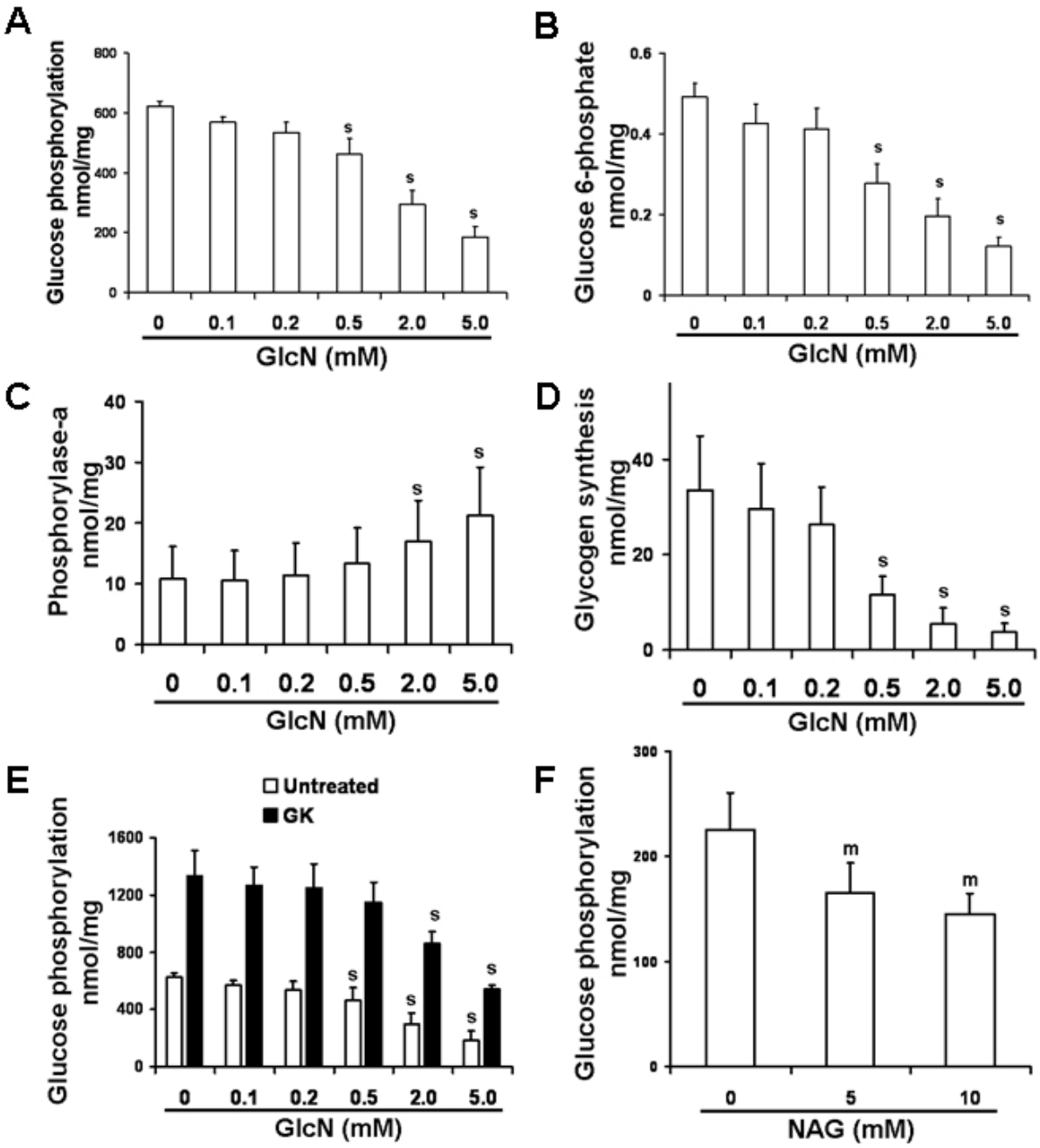


Figure 3.9 Effects of glucosamine (GlcN) and N-acetylglucosamine (NAG) on glucose phosphorylaion, glucose 6-phosphate, phosphorylase-a activity and glycogen synthesis

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone and 5 mM glucose. They were then incubated for 4 h in MEM containing 25 mM glucose and the concentrations of GlcN indicated for determination of glucose phosphorylation (A), glucose-6phosphate (B), phosphorylase-a activity (C) and glycogen synthesis (D). They were then incubated for 4 h in MEM containing 25 mM glucose, untreated or treated with vectors for expression of glucokinase (GK) (E) and the concentrations of NAG (F) and indicated for determination of glucose phosphorylation. Results are expressed as means ± SEM for 4 experiments. ^{s} $P < 0.05$ effects of glucosamine, ^{m} $P < 0.05$ effects of N-acetylglucosamine.

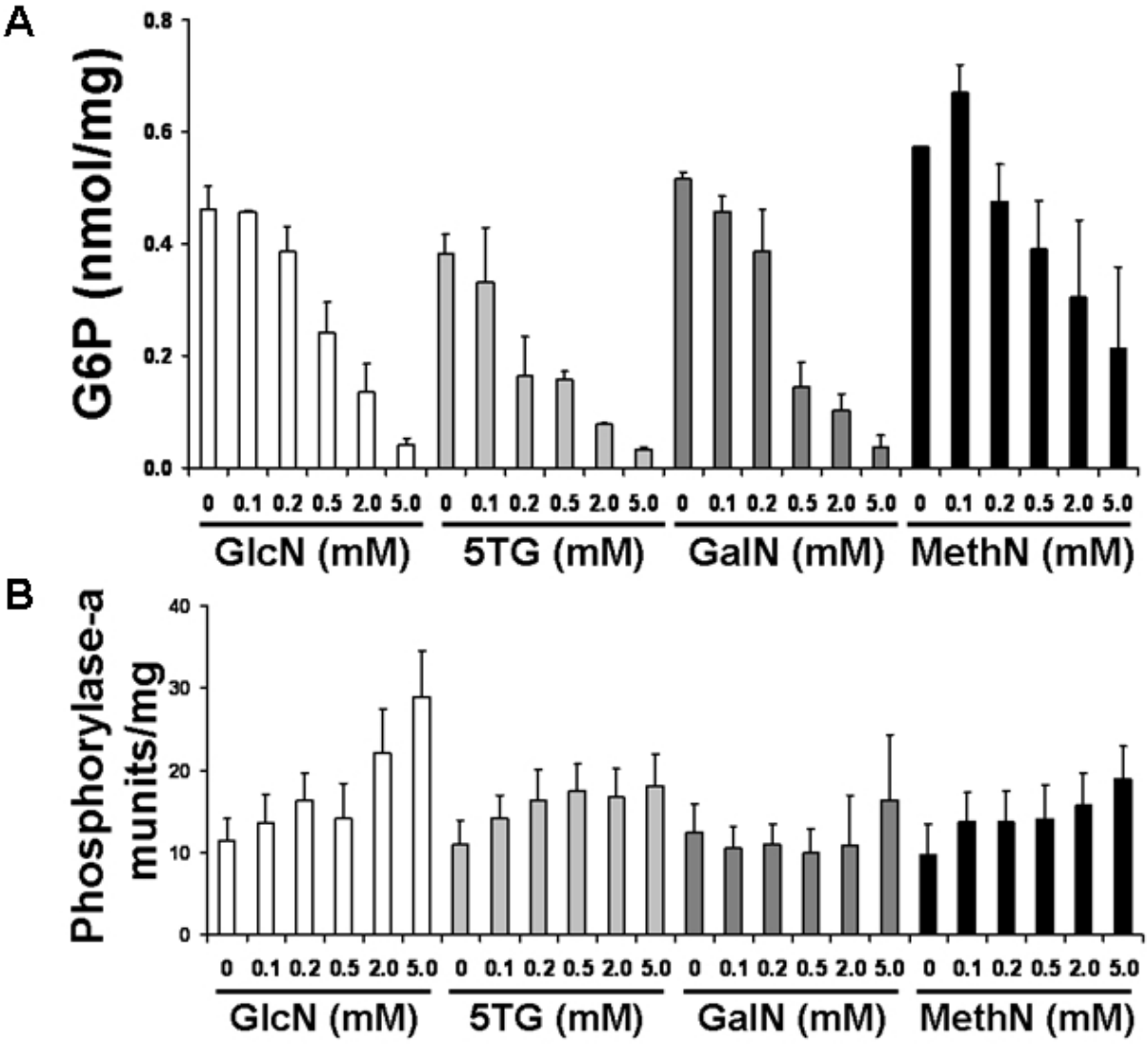


Figure 3.10 Comparison of glucosamine (GlcN) with other hexosamines and a glucokinase inhibitor

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 25 mM glucose and the concentrations of glucosamine (GlcN), 5-thioglucose (5TG), galactosamine (GalN) and methylamine (MethN) indicated for determination of glucose 6-phosphate (A) and phosphrylase-a activity (B). Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6).

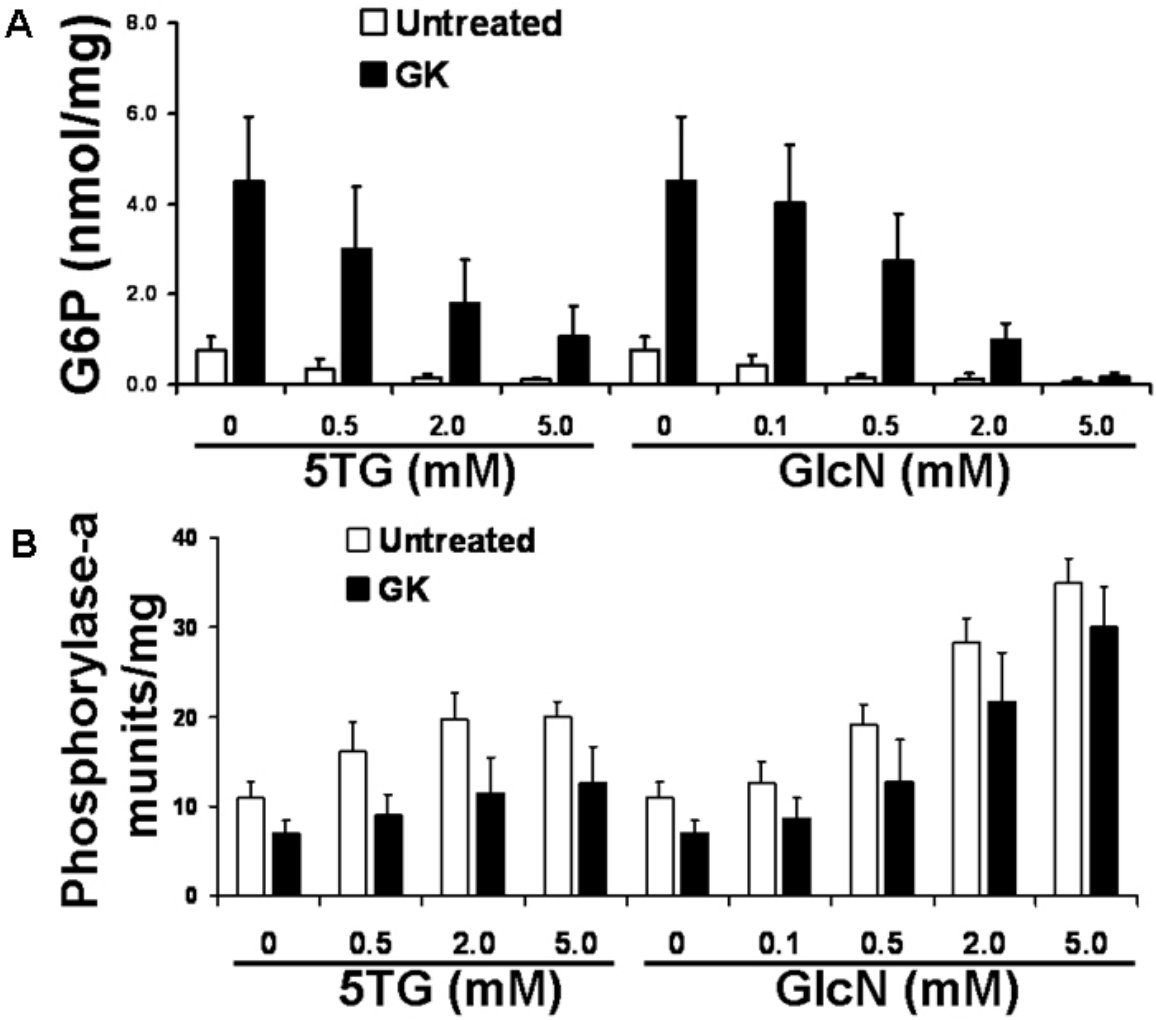


Figure 3.11 Comparison of glucosamine (GlcN) and 5-thioglucose (5TG) without or with glucokinase overexpression (GK)

Hepatocyte monolayers were untreated or treated with vectors for expression of glucokinase (GK) and pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 25mM glucose and the concentrations of 5-thioglucose (5TG) and glucosamine (GlcN), indicated for determination of glucose 6-phosphate (A) and phosphrylase-a activity (B). Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6).

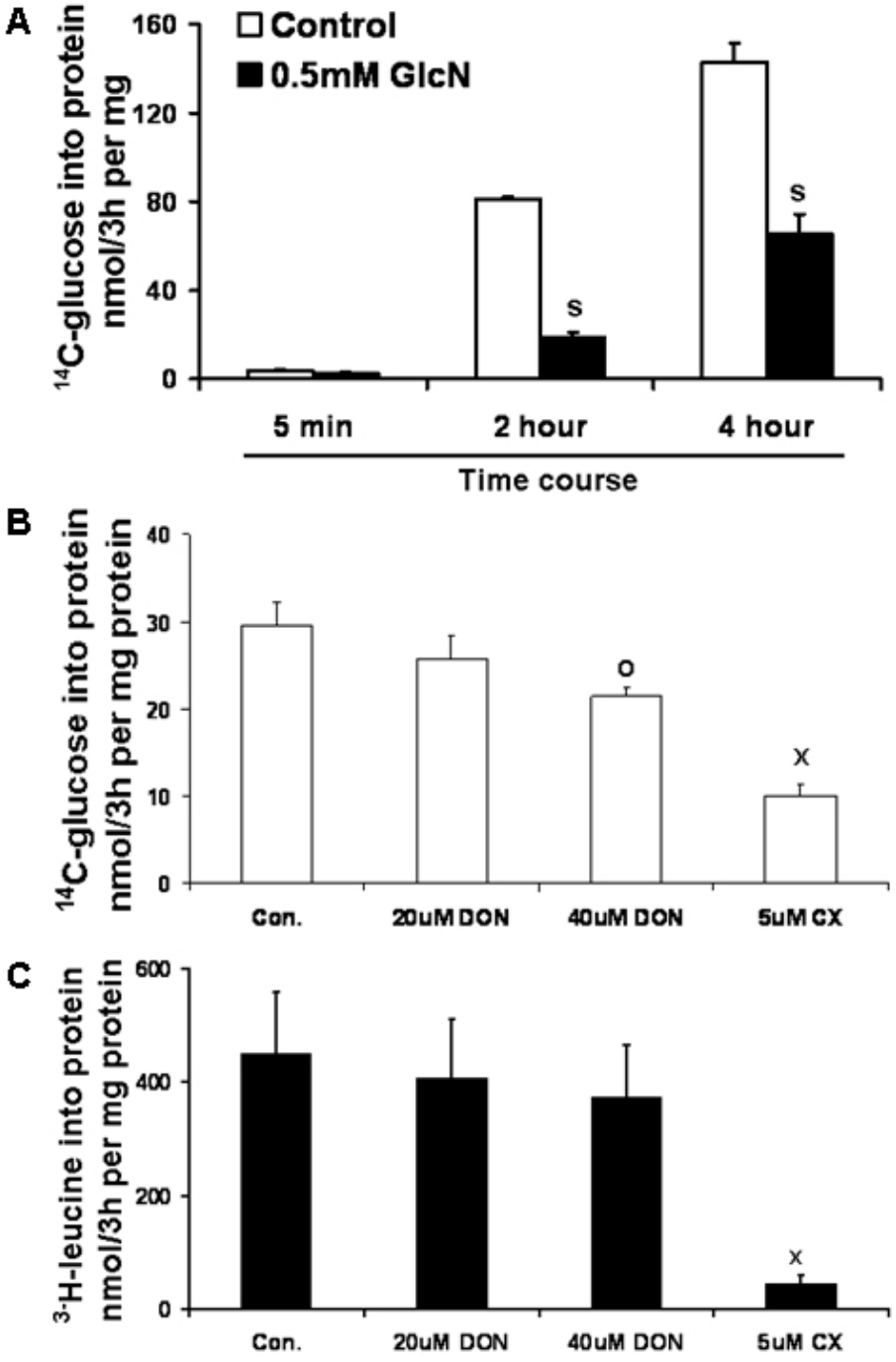


Figure 3.12 Effect of DON on ^{14}C-glucose incorporation into protein and ^{3}H-leucine incorporation into protein

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 25 mM glucose and glucosamine (GlcN), ^{14}C-glucose (6 uCi/ml) for time course (**A**). Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- 6-diazo-5-oxonorleucine (DON). They were then incubated for 4 h in MEM containing 25 mM glucose, ^{14}C-glucose (6 uCi/ml) (**B**) and ^{3}H-leucine (2 uCi/ml) (**C**) with 20 and 40 µM DON and 5 µM cycloheximide (CX). Results are expressed as means ± SEM for 4 experiments. ^{O} $P < 0.05$ effects of DON, $^{X}P < 0.05$ effects of CX, $^{S}P < 0.05$ effects of GlcN

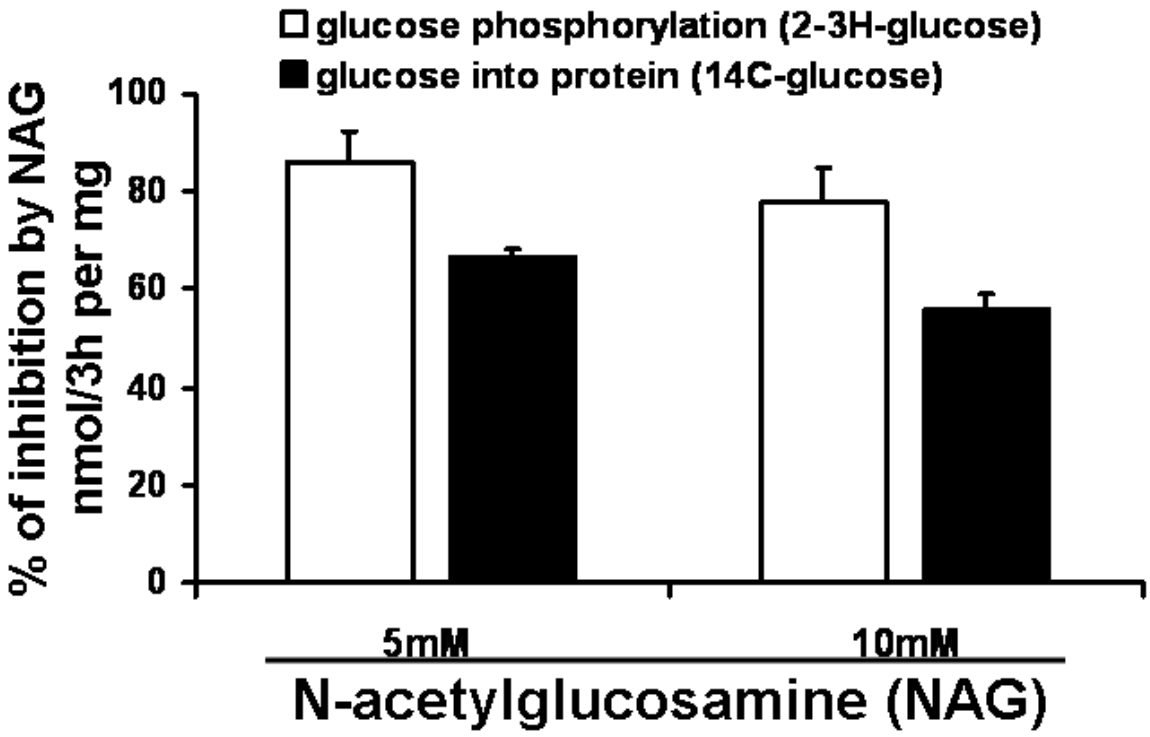


Figure 3.13 Effect of N-acetylglucosamine (NAG) on glucose incorporation into protein and glucose phosphorylation

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 25 mM glucose, ^{14}C-glucose (6 uCi/ml) (**A**) and $^{2\text{-}3}$H-glucose (3 uCi/ml) (**B**) with 5 and 10 mM N-acetylglucosamine (NAG). Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6).

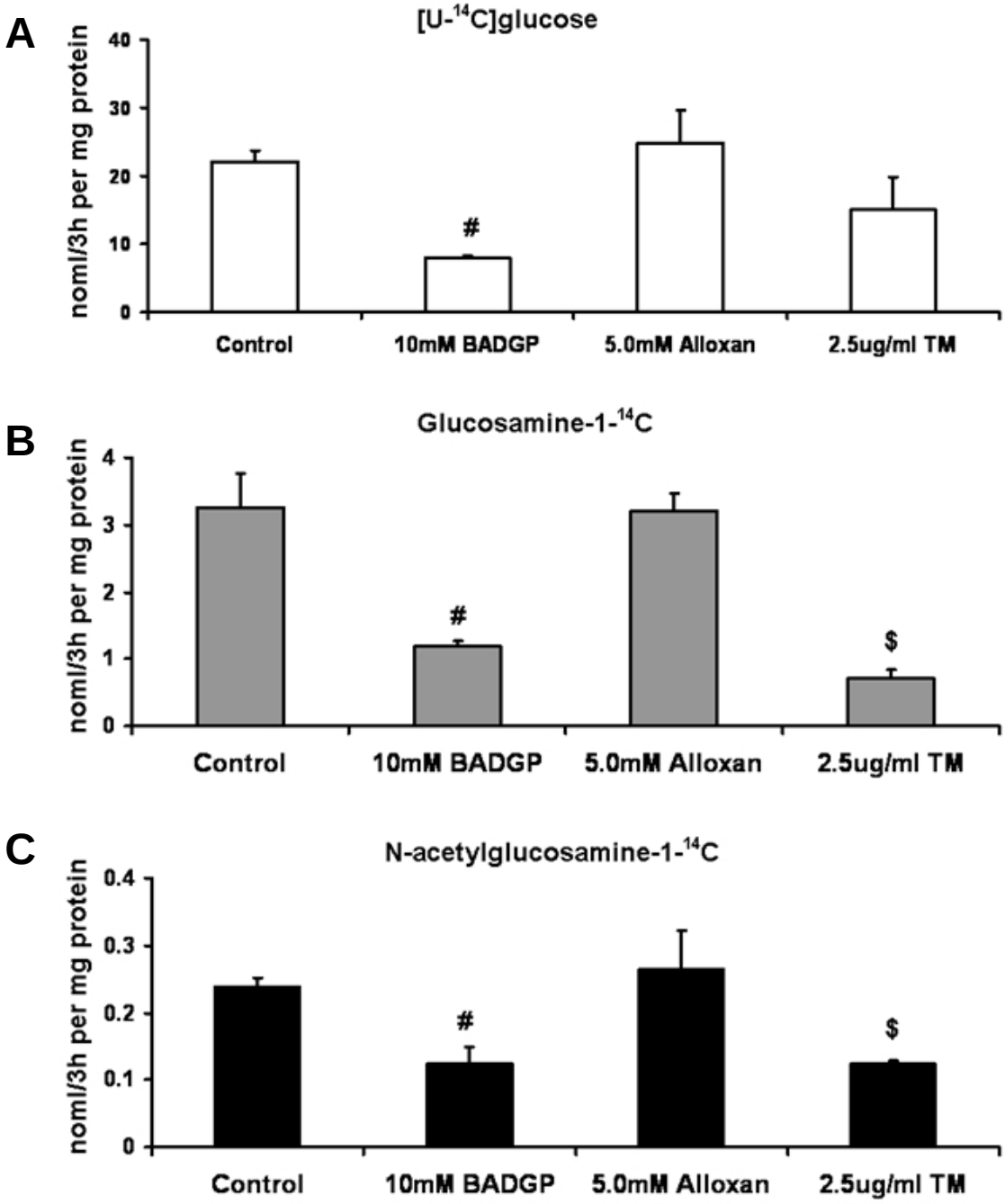


Figure 3.14 Effect of inhibitors of N-linked and O-linked glycosylation on glucose, glucosamine and N-acetylglucosamine incorporation into protein
Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM Insulin and 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 25 mM glucose, 14C-glucose (6 uCi/ml) (A), 14C-glucosamine (20uCi/ml) (B) and 14C-N-acetylglucosamine (20 uCi/ml) (C) with 10 mM benzyl-2acetamido-2-deoxy-alpha-D-galactopyranoside (BADGP), 5 mM Alloxan and Tunicamycin (TM). Results are expressed as means ± SEM for 4 experiments. $^{\#}P< 0.05$ effect of BADGP, $^{\$}P< 0.05$ effect of TM.

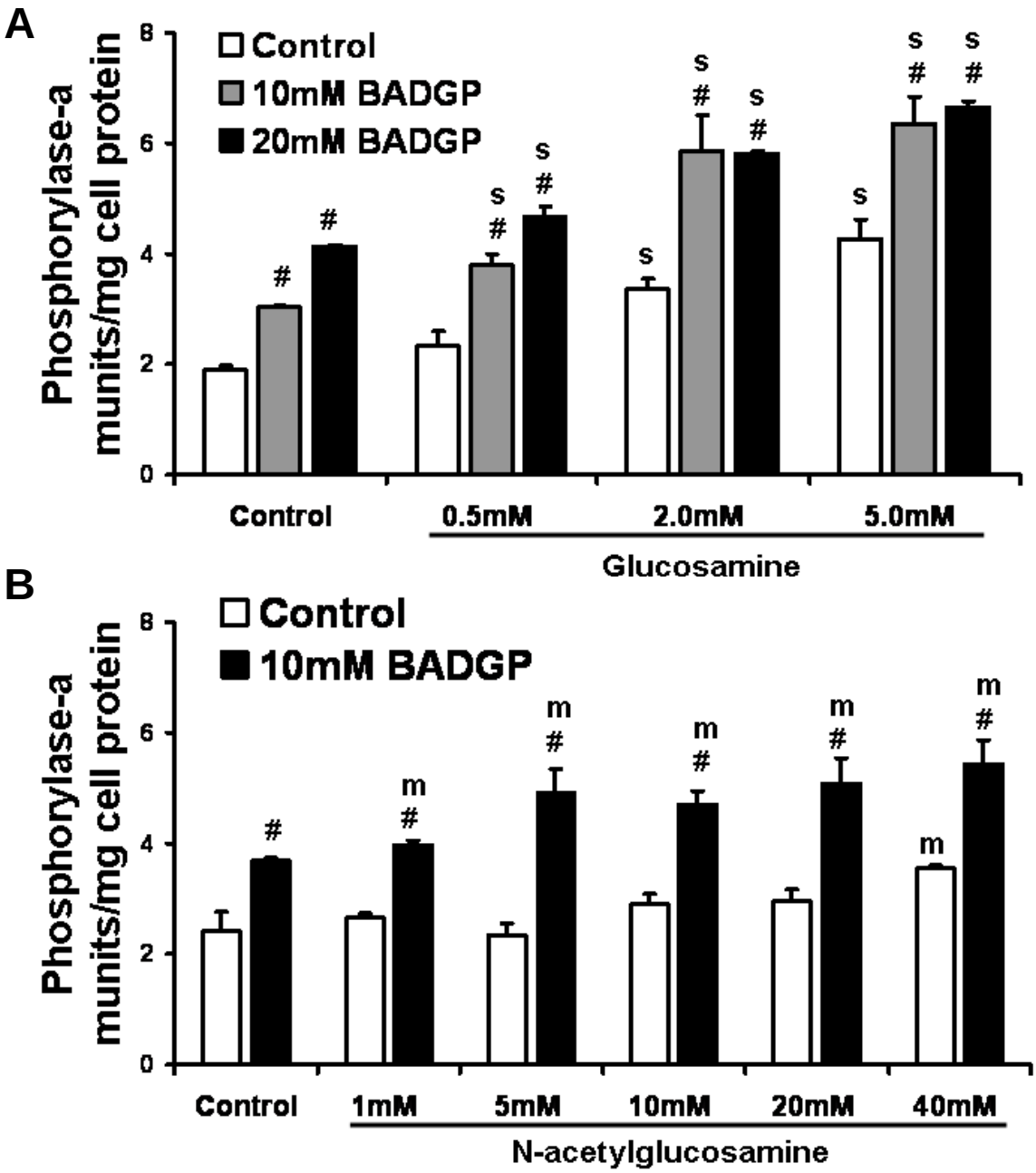


Figure 3.15 Effect of glucosamine (GlcN) and N-acetylglucosamine (NAG) without or with BADGP on glycogen phosphorylase-a activity

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM glucose and the concentrations of glucosamine (A) and N-acetylglucosamine (B), without or with 10 and 20 mM BADGP, indicated for determination of phosphrylase-a activity. Results are expressed as means ± SEM for 2 experiments, duplicate treatments (n=4).

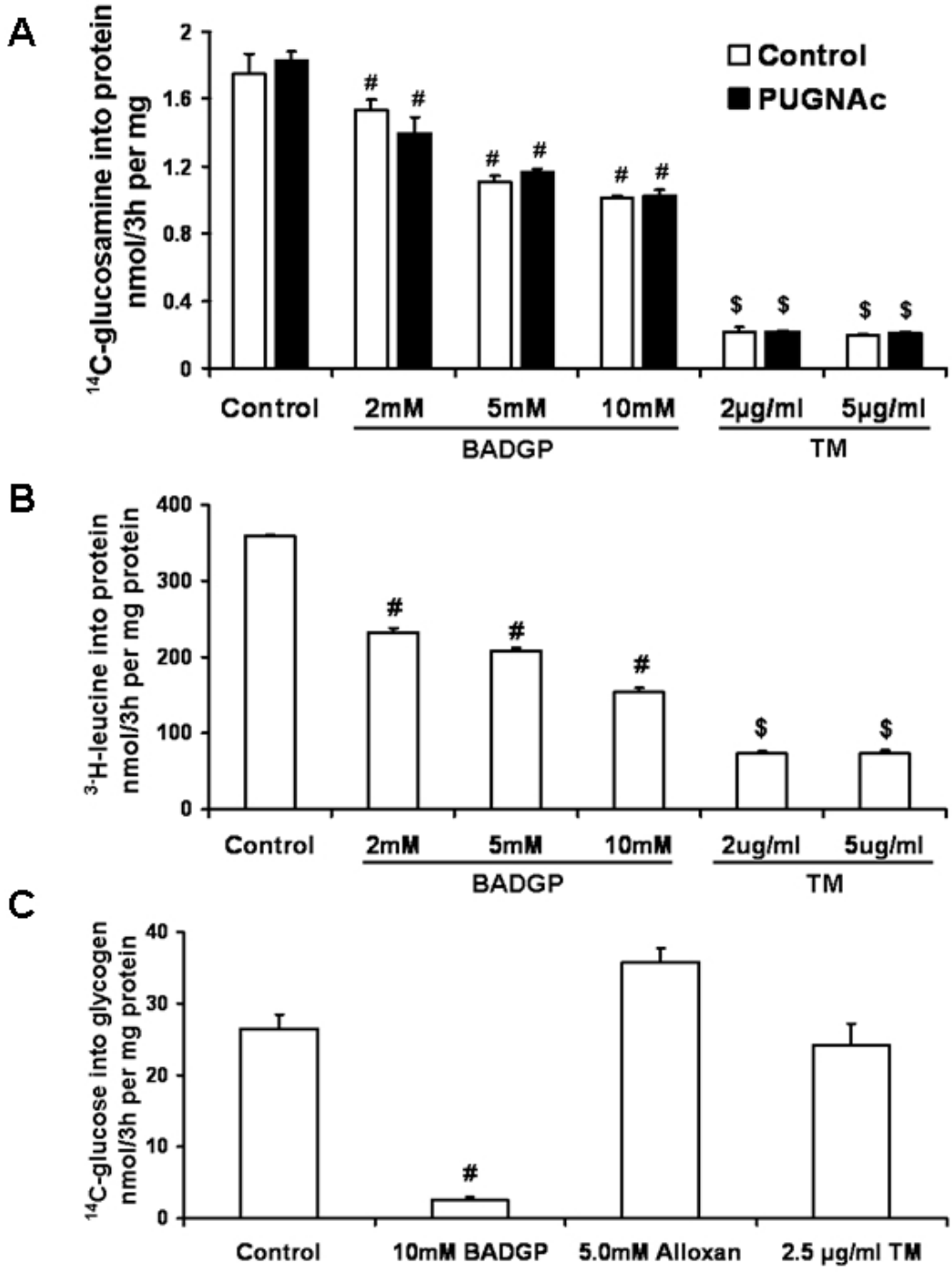


Figure 3.16 Effect of inhibitors of N-linked and O-linked glycosylation on glucose and leucine incorporation into protein or glycogen

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10nM Insulin and 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 25 mM glucose, ^{14}C-glucose (6 uCi/ml) (A,C) and ^{3}H-leucine (2 uCi/ml) (B) with 2, 5 and 10 mM benzyl-2acetamido-2-deoxy-alpha-D-galactopyranoside (BADGP), 5 mM Alloxan, 2.5 µg/ml Tunicamycin (TM) and 100 µM O-(2-acetamidO-2deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc). Results are expressed as means ± SEM for 3 experiments, duplicate treatments (n=6). $^{\#}P< 0.05$ effect of BADGP, $^{\$}P< 0.05$ effect of TM.

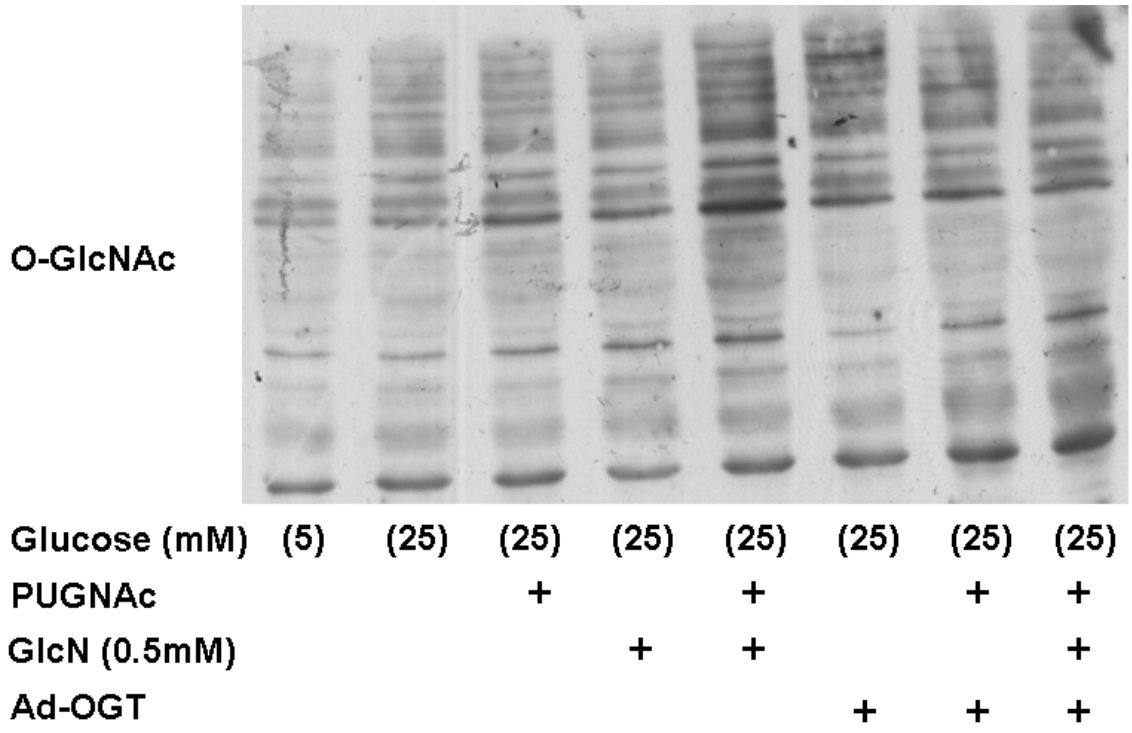


Figure 3.17 Effects of ovrexpression of OGT, PUGNAc and glucosamine (GlcN) on O-GlcNAc modification of protein

Hepatocyte monolayers were were either untreated or treated with vectors for expression of O-linked beta-N-acetylglucosamine transferase(Ad-OGT). After 18h pre-cultured in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM glucose, 25 mM glucose, 0.5mM GlcN, 100μM PUGNAc and determination of O-GlcNAc protein by immunoblotting assay. Representative of 2 experiments.

Chapter 4

The role of HBP in mediating glucose control of gene expression in hepatocytes

The role of HBP in mediating glucose control of gene expression in hepatocytes

4.1 Aims and rationale

The liver plays a key role in glucose homeostasis by two mechanisms: (i) the uptake and storage of glucose via glycogenesis and glycolysis/lipogenesis, (ii) the release of glucose via glycogenolysis and gluconeogenesis. Gene expression of the enzymes in these metabolic pathways is regulated by the hormones insulin and glucagon and also by glucose. Two mechanisms have been the focus of glucose control of gene expression. First the regulation by the transcription factor ChREBP (Carbohydrate response element binding protein) of enzymes of glycolysis and lipogenesis including L-type pyruvate kinase (L-PK), fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (Towle et al., 1997; Girard et al., 1997; Rumberger et al., 2003). ChREBP interacts with Max-like protein X (Mlx) to form a heterotetramer that binds carbohydrate responsive elements (ChoRE) in the promoters of glucose-responsive genes to increase transcription (Shih et al., 1995; Yamashita et al., 2001; Stoeckman et al., 2004). Suggested mechanisms by which glucose metabolism causes activation of ChREBP include: (i) xylulose 5-P an intermediate of the pentose phosphate pathway which causes dephosphorylation of ChREBP (Uyeda et al., 2002); (ii) glucose 6-P the first intermediate of glucose metabolism (Dentin et al., 2012); (iii) fructose 2,6-bisphosphate ($F2,6P_2$), an allosteric regulator of the enzyme of glycolysis phosphofructokinase-1 (Arden et al., 2012; Petrie et al., 2013).

The second mechanism for glucose regulation of gene expression focuses on the covalent modification of transcription factors by O-linked beta-N-

acetylglucosamine (O-GlcNAc) transferase (OGT) which uses as substrate UDP-GlcNAc generated by the HBP. The regulation of G6Pc by glucose has been shown in several studies (Massillon, 2001; Ma et al., 2006). This mechanism is often described as "glucotoxicity" because induction of G6Pc by high glucose further aggravates the hyperglycaemia (Gautier-Stein et al., 2012). A recent study proposed that this mechanism involves covalent modification by O-GlcNAc of CREB regulated transcription coactivator 2 (CRTC2), the binding protein of CREB; this was supported by the observation that GlcN is more effective than glucose at inducing G6Pc (Dentin et al., 2008).

The objective of the work in this chapter was to determine the role of HBP in mediating control of G6Pc gene expression by glucose.

4.2 Results

4.2.1 Glucose induces G6Pc irrespective of the presence of insulin or glucagon

Several studies have reported induction of G6Pc by high glucose. In all these studies the glucose stimulation was shown in the either the absence of hormones (Massillon, 2001; Dentin et al., 2008) or in the presence insulin, (Ma et al., 2006) but there have been no comparisons in different hormone conditions. The first aim was to determine the induction of G6Pc by glucose (25 mM compared with 5 mM) in the absence or presence of insulin or glucagon. Incubation of hepatocytes with 25 mM glucose, for 4 h caused a 6 fold increase in G6Pc mRNA in the absence of insulin and 3 fold increase with insulin (Fig 4.1, A). Insulin inhibited G6Pc gene expression by 60% at 5 mM glucose and by 80% with 25 mM glucose. High glucose also increased G6Pc in the presence of glucagon (Fig. 4.1B).

Because glucosamine was shown to cause greater induction of G6Pc than glucose (Dentin et al., 2008), we tested the effects of GlcN in cells treated without or with glucagon to activate CREB. However, there was no significant stimulation by 5 mM GlcN on G6Pc mRNA in these experimental conditions (Fig 4.1, B).

4.2.2 Glucosamine does not mimic the induction of G6Pc by high glucose

We next tested the effects of GlcN on G6Pc mRNA levels at different concentrations (0.5, 1, 5 and 10 mM GlcN) in comparison with 25 mM glucose in either the absence or presence of insulin (Fig. 4.2). Glucose (25 mM) increased G6Pc mRNA by 10-fold in absence of insulin or 3-fold in the presence of insulin. However, there was no significant effect of GlcN in either absence or presence of

insulin at concentrations of 0.5 to 10 mM (Fig 4.2, A and B). We next compared the effects of GlcN and N-acetylglucosamine (NAG) concentration (5, 7 and 10mM) on both NAG metabolites (Fig 4.3, A and B) and G6Pc mRNA levels (Fig 4.3, C and D). NAG unlike GlcN enters the HBP after GFAT and does not cause changes in GlcN 6-P, whereas GlcN causes a larger increase in GlcN 6-P than in NAG metabolites (Chapter 3). These experiments showed that GlcN (0.5 to 10 mM) caused a concentration-dependent accumulation of NAG-metabolites with a similar or higher effect of 0.5mM GlcN as 25mM glucose (Fig 4.3A). However, higher concentrations of GlcN (1, 5 and 10mM) caused a much larger stimulation (9-50 fold) than glucose. NAG caused a smaller increase in cellular accumulation of NAG-metabolites than GlcN and this was significant with concentrations of NAG at 10mM or higher (Fig. 4.3B). These concentrations of GlcN and NAG which significantly increase NAG metabolites to a similar or higher level than glucose did not increase G6Pc mRNA (Fig 4.3 C and D).

4.2.3 Glucosamine partially counteracts the glucose elevation of glucose 6-phosphate and Fructose 2,6-bisphosphate

The next aim was to determine the effect of GlcN in metabolism of glucose and formation of other metabolites of glucose that may be involved in G6Pc induction such glucose 6-P and $F2,6P_2$. In this study we used the lowest concentration of GlcN that increases NAG metabolites to test its effect on glucose 6-P and $F2,6P_2$ (Fig. 4.4). Incubation of hepatocytes with 25 mM glucose for 4 h caused an increase in concentration of both glucose 6-P and $F2,6P_2$ relative to 5 mM glucose. Pre-treatment of the hepatocytes with 0.5 mM GlcN partially

decreased both glucose 6-P and F2,6P_2 at 25mM glucose (Fig. 4.4). These results show that there is lowering by GlcN of glucose metabolites even at 0.5 mM.

4.2.4 OGT does not enhance the glucose induction of G6Pc

Various studies including that by (Dentin et al., 2008) and others (Yang et al., 2008) have used overexpression of OGT and OGA to test the role of O-GlcNAc modification of proteins in regulation of G6Pc and other genes (Soesanto et al., 2008). We therefore determined the effects of OGT overexpression in incubations both with 5 and 25 mM glucose. In these experiments overexpression of OGT was confirmed by immunoblotting (Fig. 4.5, A). There was no significant stimulation of G6Pc mRNA levels at either 5 or 25 mM glucose by OGT overexpression (Fig 4.5, B).

4.2.5 DON inhibits the glucose induction of G6Pc but not the induction of PTG

We next tested the effects of inhibition of GFAT with DON on G6Pc gene expression. In the previous Chapter we established the concentrations of DON and incubation times required to inhibit GFAT activity in hepatocytes, and also that DON inhibits the increase in NAG metabolites by 25 mM glucose. For these experiments it was necessary to pre-culture the hepatocytes with DON before incubation with 25 mM glucose. When the effects of 25 mM glucose were tested in cells without or with preculture with DON, glucose (25 mM) caused 4-fold stimulation and this effect was partially but significantly inhibited by DON (Fig 4.6, A). However, there was no significant effect of DON on GAPDH mRNA levels (Fig 4.6, B). To test whether the inhibitory effect of DON is due to

lowering of NAG metabolites (by inhibition of the HBP) or non-specific effects independent of NAG metabolites, we used a low concentration of GlcN (0.5 mM) which increases NAG metabolites by entry after GFAT, on mRNA expression of various genes (G6Pc, TXNIP, L-PK and PTG). The result of this study shows that high glucose (25 mM) significantly stimulated gene expression of all 4 genes and treatment with DON inhibited the glucose stimulation of G6Pc and TXNIP but not on L-PK and PTG. In the presence of 0.5 mM GlcN the inhibitory effect of DON on G6Pc was significantly attenuated (Fig. 4.7, A) and in the case of L-PK, GlcN stimulated in the presence of DON (Fig. 4.7, C). These results suggest that the effect of DON on gene expression is probably at least in part due to NAG metabolites.

4.2.6 NAG and GlcN partially reverse the effects of DON on G6Pc

The inhibitory effect of GlcN on L-PK and G6Pc gene expression at 25 mM glucose (Fig 4.7 A and C), suggests a counter-regulatory effect of GlcN on glucose activation. To test whether this effect of GlcN may be due to GlcN 6-P and not NAG metabolites, we compared the effect of 0.5 mM GlcN with NAG which does not increase GlcN 6-P. In this experiment there was a small inhibition of the stimulation by 25 mM glucose by GlcN (Fig 4.8, A) but not by NAG (Fig 4.8, B). However, both GlcN and NAG partially reversed the inhibition of G6Pc gene expression by DON. This confirms: first, that the inhibitory effect of DON at 25 mM glucose is in part due to the lowering of NAG metabolites (because it is reversed by both GlcN and NAG); secondly that GlcN but not NAG has an inhibitory effect at 25 mM glucose suggesting that this effect is probably due to GlcN-6-P rather than NAG metabolites.

4.2.7 DON counteracts induction of G6Pc by ChREBP; DON inhibits ChREBP translocation

We next tested the effect of overexpression of ChREBP with an adenoviral vector on the glucose-induction of G6Pc. The available antibodies for ChREBP did not detect any immunoactivity in cells not treated with the adenovirus. In cells treated with the adenoviral vector for overexpression of ChREBP there was increased 40-fold in ChREBP mRNA (Fig. 4.9, A). Overexpression of ChREBP increased G6Pc mRNA at both 5 mM and 25 mM glucose but the stimulatory effect of ChREBP overexpression was much greater at 25 mM glucose (Fig 4.9, B). Immunoblotting experiments showed weak immunoactivity in cells treated with the adenoviral vector (Fig. 4.9, C). The induction of G6Pc by ChREBP at 25 mM glucose was inhibited by DON. In addition, we tested whether GlcN (0.5 mM) reverses this effect of DON with overexpression of ChREBP. In these experiments GlcN partially reversed the inhibition of G6Pc by DON with ChREBP overexpression (Fig 4.10). Immunoblotting for ChREBP in the overexpression experiments showed lower immunoactivity in DON-treated cells. We next tested whether DON affects the translocation of ChREBP between the nucleus and cytoplasm under low and high glucose from immunostaining for ChREBP with the Novus antibody (Fig. 4.11). For these experiments it was necessary to overexpress ChREBP because the endogenous protein was not detectable with the antibody. The results showed greater nuclear staining for ChREBP after 1 h incubation with 25 mM glucose and this effect was significantly inhibited in cells pre-treated with DON (Fig 4.11). These results suggest that the effect of DON on gene expression may be in part on ChREBP activation.

4.2.8 Substrate stimulation of G6Pc: DON does not affect the increase in fructose 2,6-bisphosphate

Previous studies suggested a role for substrates such as glucose 6-P or xylulose 5-P in ChREBP activation through activation of a type 2A phosphatase that dephosphorylates ChREBP (Uyeda et al., 2002). The role of xylulose 5-P metabolites was demonstrated from studies using xylitol, that is metabolised to xylulose 5-P, Masillon study showed a higher induction of G6Pc by xylitol compared with 25 mM glucose (Massillon, 2001). Work from our laboratory identified a role for and $F2,6P_2$ in regulation of ChREBP target genes by glucose and also in the glucose stimulation of ChREBP translocation from the cytoplasm to the nucleus (Arden et al., 2012). Our next aim was therefore to test whether the inhibition of G6Pc gene expression or ChREBP translocation by DON can be explained by an effect of DON on the metabolites involved in ChREBP activation. Therefore, we first tested the effect of DON on G6Pc gene expression with various substrates (Fig. 4.12A). The expression of G6Pc mRNA was stimulated (3-fold) by xylitol (5 mM) and (6-fold) by 25 mM glucose and these effects were additive resulting in a much larger induction of G6Pc (16-fold) in the combined presence of 5 mM xylitol and 25 mM glucose. The stimulation by 25 mM glucose was also enhanced by overexpression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-WT) which increases $F2,6P_2$ (10-fold) and also by S4048 (19-fold), an inhibitor of transport of glucose 6-P to the endoplasmic reticulum (ER), which causes a large increase in both glucose 6-P and $F2,6P_2$. Treatment with DON did not affect the stimulation of G6Pc mRNA by xylitol but it inhibited the stimulation by 25 mM glucose either alone or in combination with xylitol, S4048 and PFK-WT overexpression. We compared the

effects of DON on G6Pc with the expression of PTG, which shows similar but smaller fold induction of expression by substrates as G6Pc. PTG which was recently shown to be a target for Mlx-MondoA rather than ChREBP-Mlx (Petrie et al., 2013), unlike the inhibition of G6Pc mRNA, DON did not inhibit the substrate stimulation of PTG mRNA (Fig 4.12B).

4.2.9 PFK-KD blocks the glucose induction but not the elevation in NAG metabolites

The stimulation of G6Pc by glucose is suggested to be due to $F2,6P_2$ because lowering of this metabolite with a kinase-deficient variant of PFK2/FBPase-2 (PFK-KD) inhibits the stimulation by high glucose. We therefore tested whether PFK-KD also affects NAG metabolites. Treatment of hepatocytes with 25mM glucose caused an increase in glucose 6-P, $F2,6P_2$, NAG metabolites and G6Pc mRNA (Figs 4.13, A-D). Treatment with PFK-KD lowers G6Pc mRNA and $F2,6P_2$ but not glucose 6-P or NAG metabolites, suggesting that the effect of PFK-KD cannot be explained by changes in glucose 6-P or NAG- metabolites.

4.2.10 DON does not affect fructose 2,6-bisphosphate

We next tested whether the effect of DON can be explained by a lowering of $F2,6P_2$ (Fig 4.14) Treatment with DON lowered G6Pc mRNA and NAG metabolites but not $F2,6P_2$, indicating that the effect of DON is not explained by lowering in $F2,6P_2$.

4.2.11 ChREBP and FOXO1 mRNA gene expression

We also tested whether the effect of DON can be explained by changes in gene expression of ChREBP and FOXO1 (Fig 4.15). There was no significant effect of DON on either ChREBP or FOXO1 mRNA levels.

4.3 Discussion

Hepatic enzymes involved of glycolysis and lipogenesis are induced by both insulin and elevated glucose (Uyeda et al., 2002, Towle, 2005), whereas the gluconeogenic enzyme G6Pc is repressed by insulin but induced by glucose. The induction of enzymes of glycolysis and lipogenesis by high glucose is mediated by the transcription factors ChREBP and Mlx, which bind as a heterotetramer (Towle, 2005). This mechanism is sometimes described as an adaptive mechanism for energy storage in accordance with the "Thrifty Genotype Hypothesis" (Uyeda et al., 2002). The induction of G6Pc by high glucose is also at least in part mediated by ChREBP and Mlx (Arden et al., 2011). This mechanism is often described as "glucotoxicity" because induction of G6Pc by high glucose further aggravates the hyperglycaemia (Gautier-Stein et al., 2012). However, an alternative hypothesis to explain the induction of G6Pc by high glucose is that it represents a mechanism for intrahepatic homeostasis of inorganic phosphate to protect the liver from ATP depletion and steatosis (Arden et al., 2012).

The results of the present study show increased G6Pc mRNA levels by glucose in both the absence and the presence of insulin confirming an insulin-independent mechanism for glucose-induction of G6Pc in hepatocytes. The aim of the present study was to determine whether the increase in G6Pc mRNA by glucose is mediated by the HBP. To test this we used two experimental approaches: incubation with concentrations of glucosamine and NAG to increase the concentration of NAG metabolites and inhibition of GFAT with DON. A recent study published before the start of this project had reported a very large induction of G6Pc by GlcN (10 mM), as evidence for the HBP.

The experiments with GlcN and NAG which were tested over a wide range of concentrations and incubation conditions did not provide evidence for induction

of G6Pc by an increase in NAG metabolites. GlcN was tested in both the absence and presence of insulin and without or with glucagon in the pre-culture or final incubation. The concentrations of GlcN and NAG used were confirmed to increase NAG metabolites by a similar or greater extent than high glucose concentration. The lack of increase in G6Pc is not consistent with the recent study (Dentin et al., 2008) which reported a greater induction of G6Pc by 10 mM GlcN than by 25 mM glucose (5-fold vs 2-fold). Possible explanations for the lack of stimulatory effect of GlcN on G6Pc gene expression are that it inhibits metabolism of glucose and formation of other metabolites of glucose that are involved in G6Pc induction such as glucose 6-P and $F2,6P_2$ (Fig. 4.4). GlcN is a glucokinase inhibitor and inhibits glucose phosphorylation and elevation in glucose 6-P caused by high glucose (Chapter 3). This study therefore used a range of concentration of GlcN, but significant lowering of both glucose 6-P and $F2,6P_2$ was also observed at the lowest GlcN concentration (0.5 mM) that raises NAG metabolites.

Using the second approach of inhibition of GFAT with a concentration of DON that counteracts the increase in NAG metabolites by high glucose, there was clear evidence for inhibition of the glucose stimulation by at least 50% and in some experiments up to 70%. To test whether the effect of DON may be due to non-specific effects unrelated to lowering of NAG metabolites low concentrations of glucosamine or NAG were used to reverse the lowering of NAG metabolites by DON (Figs. 4.7, 4.8, 4.10). These experiments showed that the inhibition by DON was in part reversed by GlcN or NAG. This suggests that the glucose stimulation of G6Pc may be at least in part mediated by flux through the HBP, but the inhibition by DON may also in part involve additional mechanisms that are not reversed by NAG metabolites. For these experiments GlcN was used at a

concentration of 0.5 mM to minimise the inhibition of glucose phosphorylation (Fig. 3.7). However, even at this low concentration, GlcN tended to lower G6Pc mRNA at high glucose. Interestingly a study that determined G6Pc mRNA by Northern Blot showed that 2 mM GlcN inhibited the induction of G6Pc by 25 mM glucose (Massillon, 2001). This inhibition by GlcN of the glucose stimulation may be explained by the lowering by GlcN of metabolites formed from glucose that are involved in ChREBP activation and translocation.

Previous studies have suggested that the glucose induction of ChREBP target genes is mediated by glucose 6-P (Dentin et al., 2012), xylulose 5-P (Kabashima et al., 2003) or $F2,6P_2$ (Arden et al., 2012). In this study overexpression of ChREBP caused an increase in G6Pc at both 5mM glucose and 25mM glucose suggesting involvement of ChREBP in G6Pc induction. This was confirmed in other experiments by ChIP assays which showed glucose dependent recruitment of ChREBP to the G6Pc promoter (Petrie et al., 2013). In this study we confirmed that DON does not lower $F2,6P_2$ indicating that the inhibition of G6Pc by DON is not explained by this mechanism. This study also confirmed that lowering of $F2,6P_2$ by expression of PFK-KD lowers G6Pc mRNA but does not lower either glucose 6-P or NAG metabolites. We can therefore conclude that the glucose induction of G6Pc involves at least two independent mechanisms the elevation in $F2,6P_2$ and also increased flux through the HBP which is also necessary for induction of G6Pc.

A comparison of the effects of DON on the mRNA levels of various genes showed no significant effect of DON on either PTG which is a MondoA target gene or on L-PK which is a ChREBP target gene (Figs. 4.6 and 4.11). One possible explanation is that the effect of DON is not mediated through ChREBP but through other transcription factors. Another possible explanation is that

because glucose causes a larger stimulation of G6Pc than L-PK, additional transcription factors may be involved in the G6Pc induction acting separately or synergistically with ChREBP and DON may affect the interaction between ChREBP and other transcription factors. The experiments on ChREBP translocation showed that DON at least in part inhibits ChREBP translocation to the nucleus, suggesting a possible mechanism for the inhibitory effect of G6Pc mRNA.

4.4 Summary

This study has shown that:

- High glucose concentration (25 mM vs 5 mM) induces G6Pc both in the absence of hormones and also in the presence of insulin or glucagon.

- Elevation of NAG metabolites with glucosamine or N-acetylglucosamine did not mimic the effect of high glucose on G6Pc mRNA and overexpression of OGT also did not induced G6Pc mRNA.

- Inhibition of HBP flux with a GFAT inhibitor counteracted glucose-induced and ChREBP-induced G6Pc gene expression and this effect was in part reversed by treatment with glucosamine. This suggests a role for the HBP flux in mediating glucose induction of G6Pc expression.

- Stimulation of G6Pc by glucose is inhibited by both lowering of $F2,6P_2$ with PFK-KD and also by DON and these treatments are each selective for $F2,6P_2$ and NAG metabolites respectively. This indicates that at least two independent metabolite signalling pathways are involved in the glucose induction of G6Pc: elevation in $F2,6P_2$ and flux through the HBP

- Although DON inhibits glucose induced G6Pc mRNA expression it does not inhibit the induction of either L-PK which is a ChREBP target gene or PTG which is a MondoA target gene. This suggests that additional regulators may be involved in glucose induction of G6Pc. This is supported by the much larger induction of G6Pc by glucose compared with L-PK or PTG.

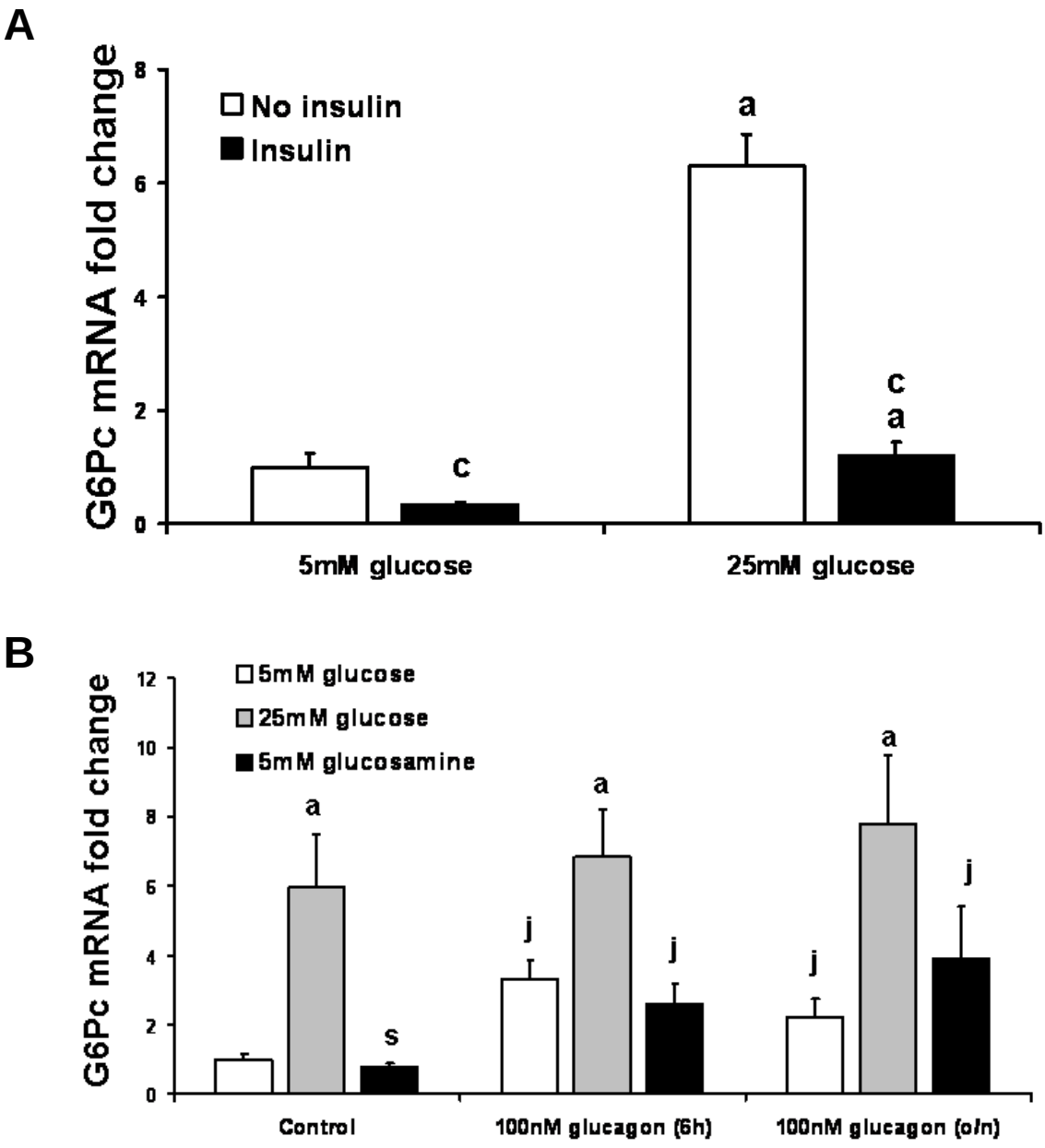


Figure 4.1 Effects of insulin on G6Pc mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose, with or without 10 nM insulin (A) and 5 and 25 mM glucose and 5 mM glucosamine, with 6 h and overnight (o/n) glucagon (B), indicated for determination of G6Pc mRNA levels. Mean ± SEM 8-12 experiments, duplicate treatments (n=16-24). $^{a}P< 0.05$ effect of glucose, $^{C}P< 0.05$ effect of insulin, $^{S}P< 0.05$ effect of glucoseamine and $^{j}P< 0.05$ effect of glucagon.

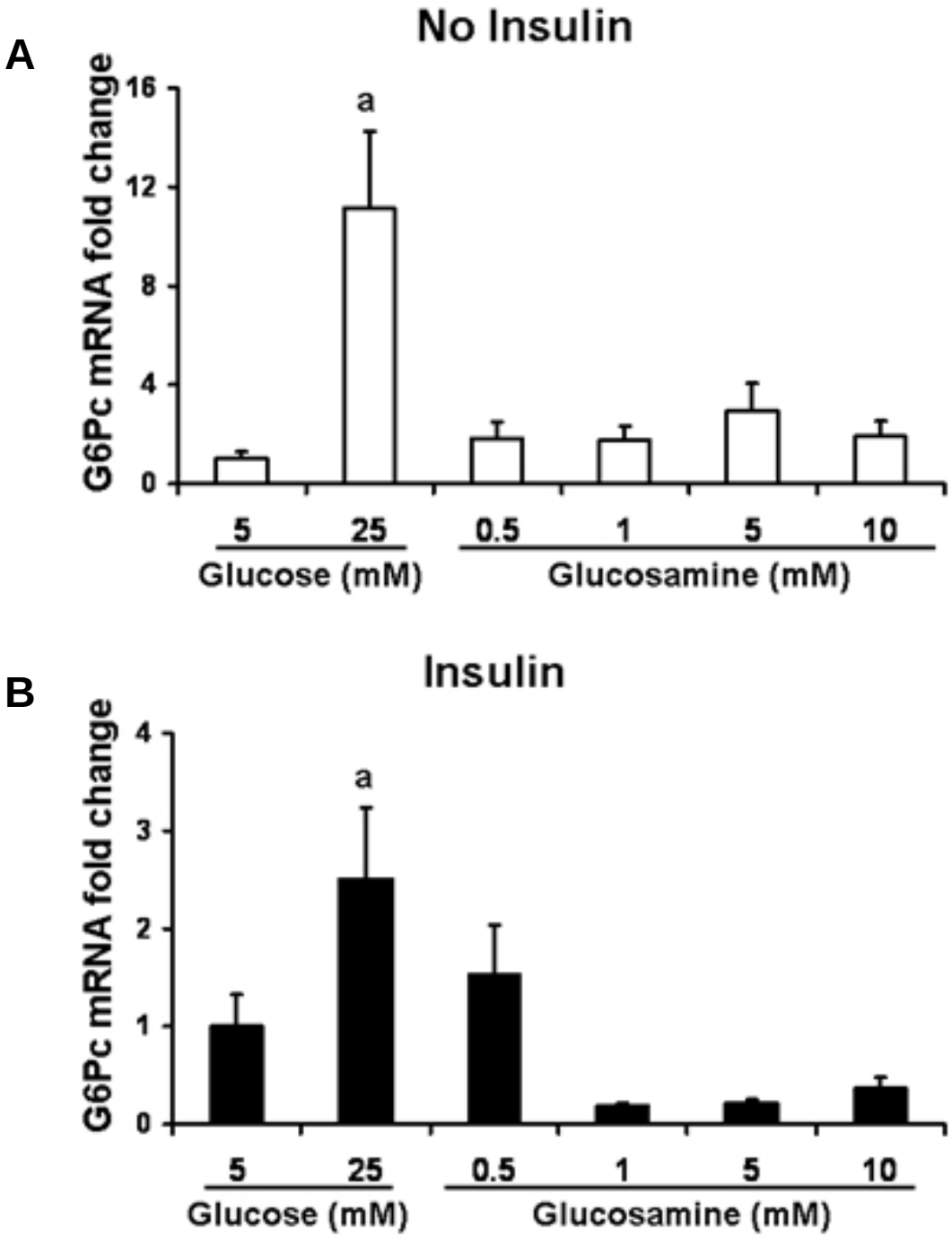


Figure 4.2 Effects of glucosamine (GlcN) on gene expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose and 0.5, 1, 5 and 10 mM glucosamine (GlcN) without insulin (A) and with insulin (B), indicated for determination of G6Pc mRNA levels. Results are expressed as means ± SEM 3 experiments, duplicate treatments (n=6). $^{a}P<0.05$ effect of glucose.

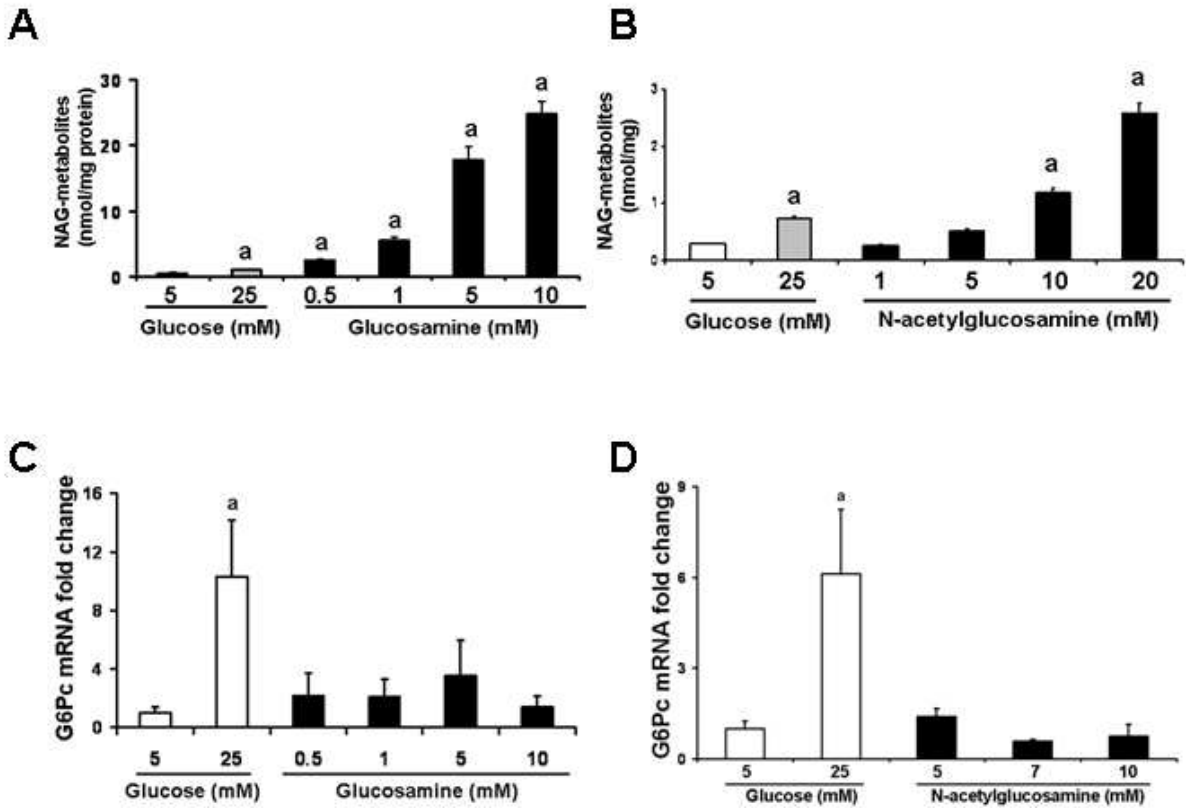


Figure 4.3 Effects of glucosamine (GlcN) and N-acetylglucosamine (NAG) on gene expression and NAG-metabolites

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose and 0.5, 1, 5 and 10 mM glucosamine (GlcN) (A, C) and 1, 5, 7, 10 and 20 mM N-acetylglucosamine (NAG) (B, D), indicated for determination of NAG-metabolites (A, B) and G6Pc mRNA levels (C, D). Results are expressed as means ± SEM 3-6 experiments, duplicate treatments (n=6-12) . $^{a}P<0.05$ effect of glucose.

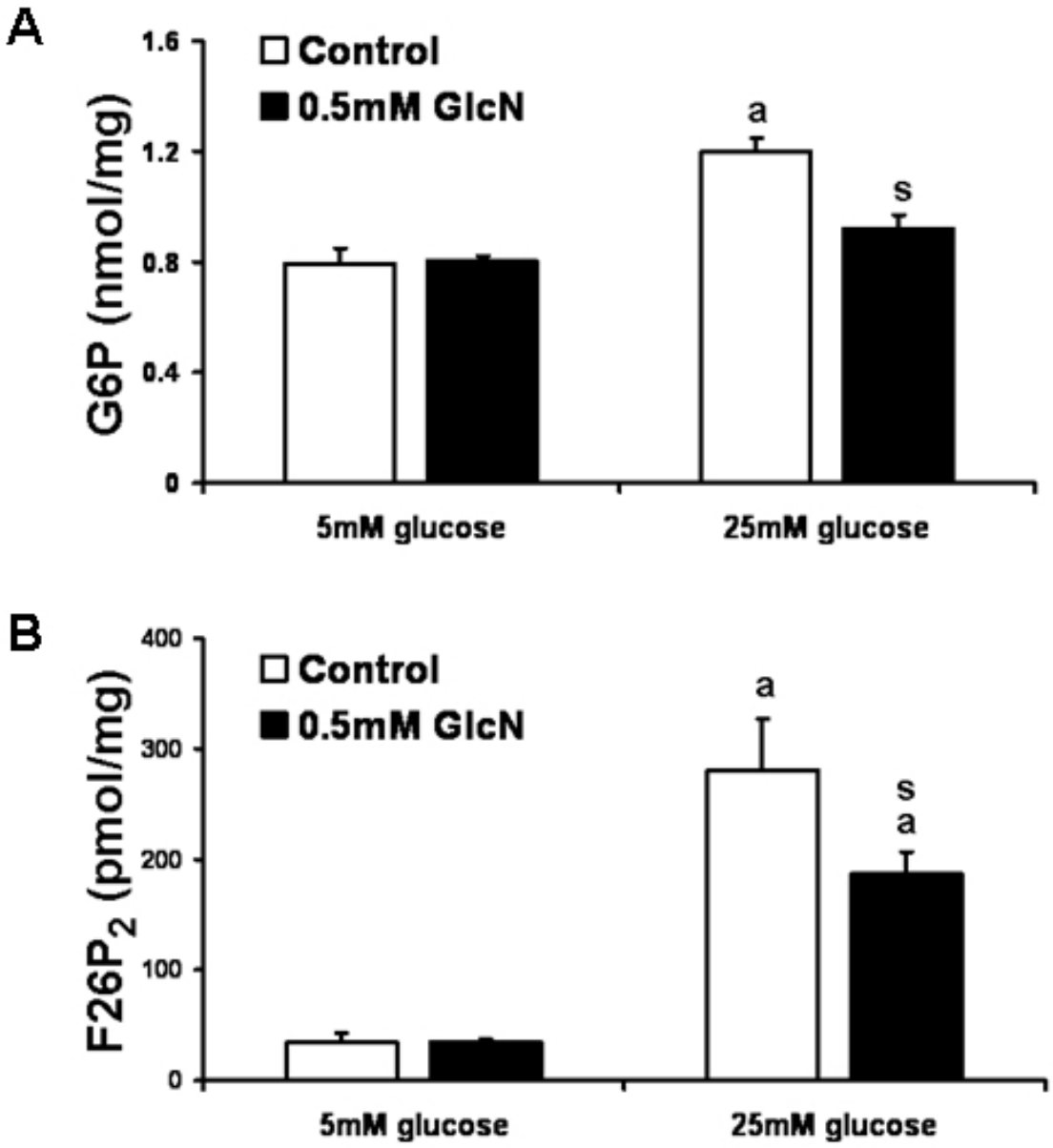


Figure 4.4 Effect of glucosamine on glucose 6-P and fructose 2,6-bisphosphate

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 0.5 mM glucosamine (GlcN) and indicated for determination of glucose 6-P (A) and $F2,6P_2$ (B). Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12) , $^{a}P< 0.05$ effect of glucose, $^{S}P< 0.05$ effect of GlcN.

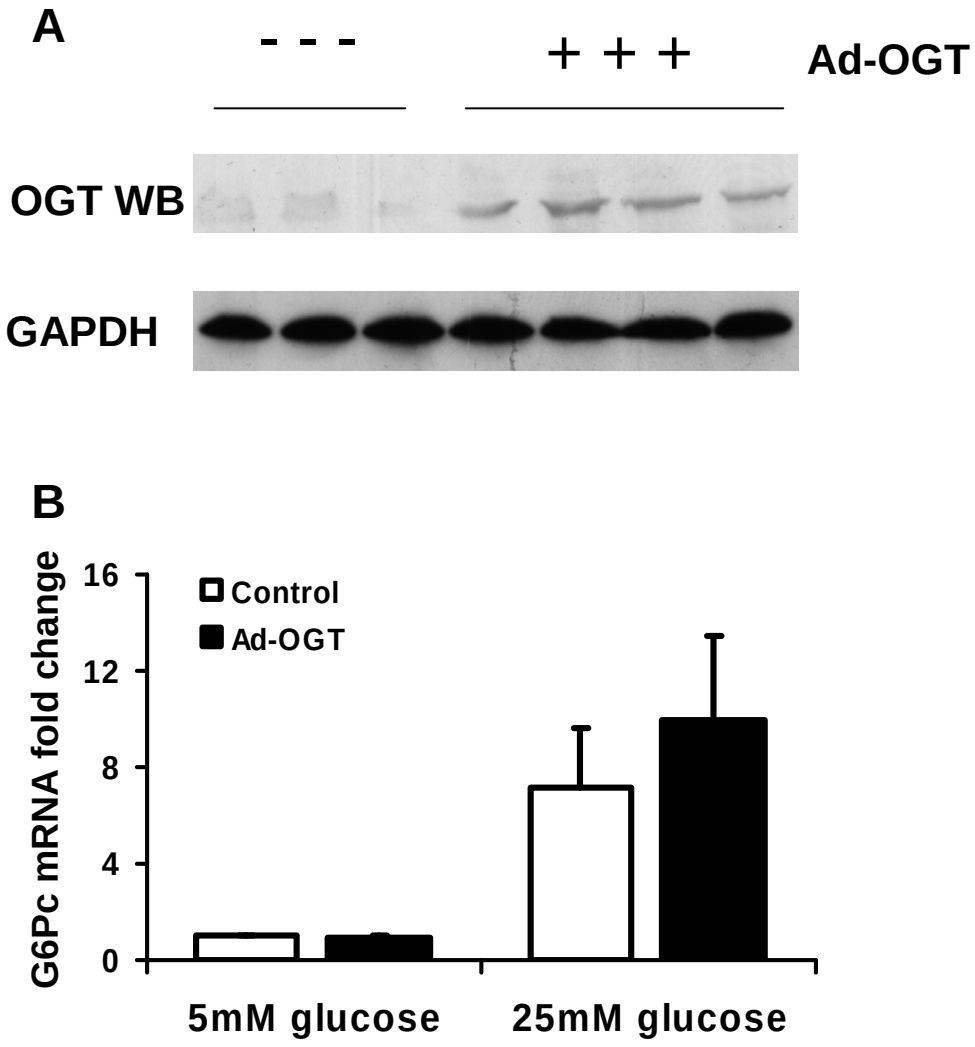


Figure 4.5 Effects of OGT on G6Pc mRNA expression

Hepatocyte monolayers were untreated or treated with vectors for expression of OGT and pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM glucose, 25 mM glucose and determination of OGT protein expression by immunoblotting assay (A) and G6Pc mRNA levels (B). Mean ± SEM 4 experiments, duplicate treatments (n=8).

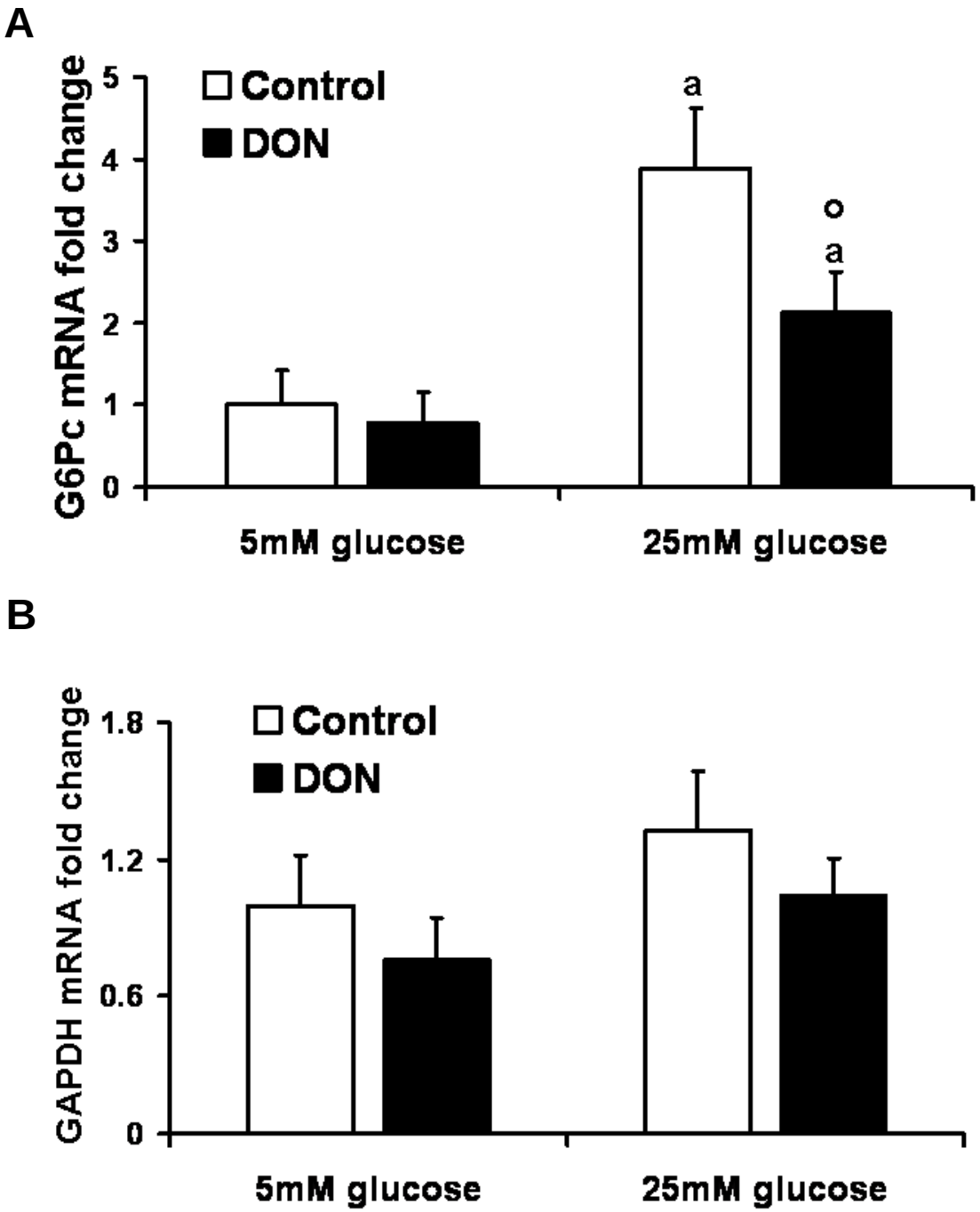


Figure 4.6 Effects of 6-diazo-5-oxonorleucine (DON) on gene expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose, with 40μM DON and indicated for determination of gene expression of (A)G6Pc, (B) GAPDH. Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12), $^{a}P< 0.05$ effect of glucose, $^{O}P< 0.05$ effect of DON.

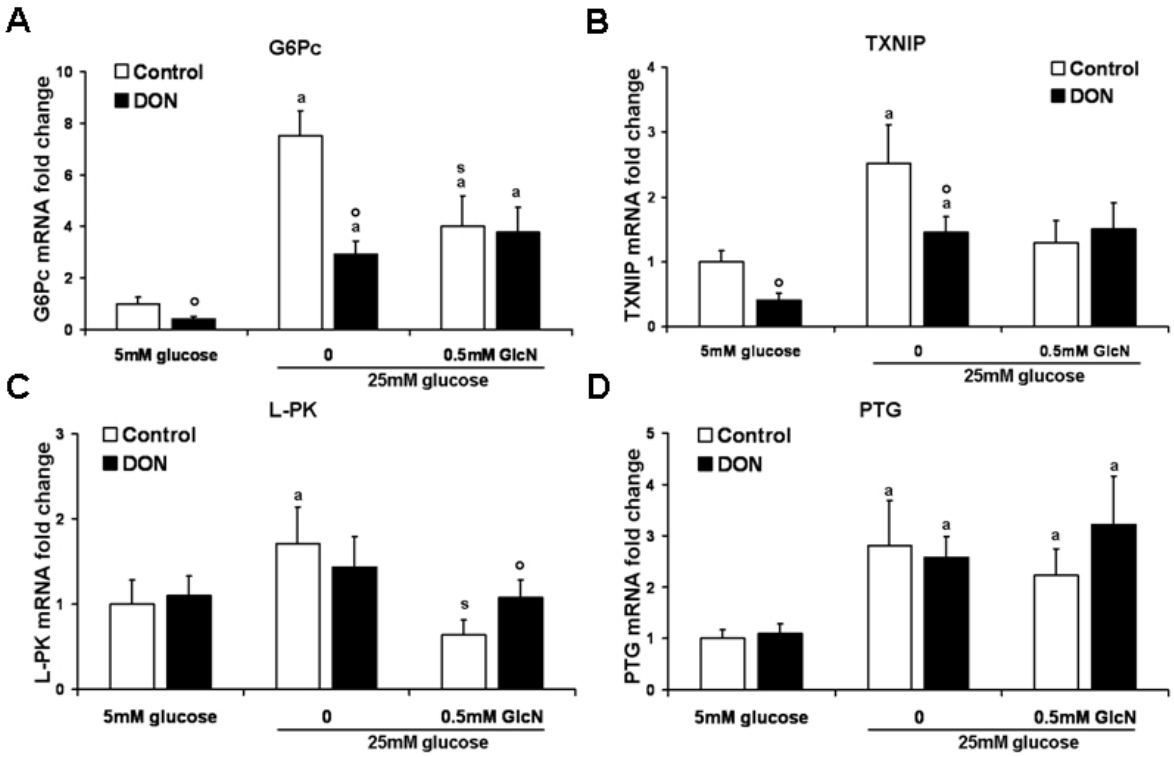


Figure 4.7 Effects of 6-diazo-5-oxonorleucine (DON) and glucosamine (GlcN) on gene expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 0.5 mM glucosamine (GlcN), with 40 µM DON and indicated for determination of gene expression of (A) G6Pc, (B) TXNIP, (C) L-PK and (D) PTG. Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12), $^{a}P< 0.05$ effect of glucose, $^{o}P< 0.05$ effect of DON $^{S}P< 0.05$ effect of GlcN.

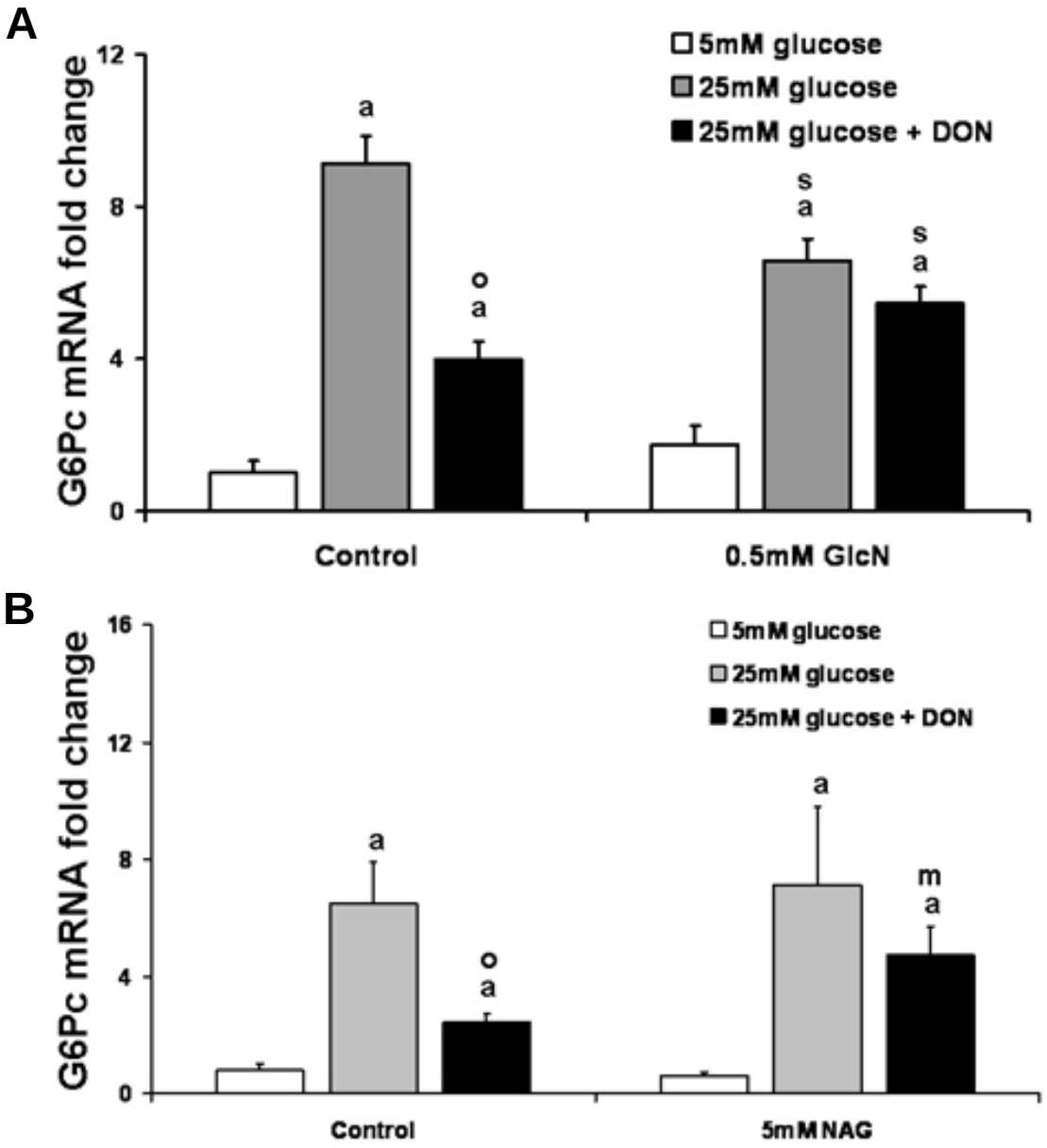


Figure 4.8 Effects of 6-diazo-5-oxonorleucine (DON), glucosamine (GlcN) and N-acetylglucosamine (NAG) on gene expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 0.5 mM glucosamine (GlcN) (A), 5 mM N-acetylglucosamine (NAG), with 40 µM DON and indicated for determination of gene expression of G6Pc. Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12), $^{a}P< 0.05$ effect of glucose, $^{o}P< 0.05$ effect of DON $^{s}P< 0.05$ effect of GlcN $^{m}P< 0.05$ effect of NAG.

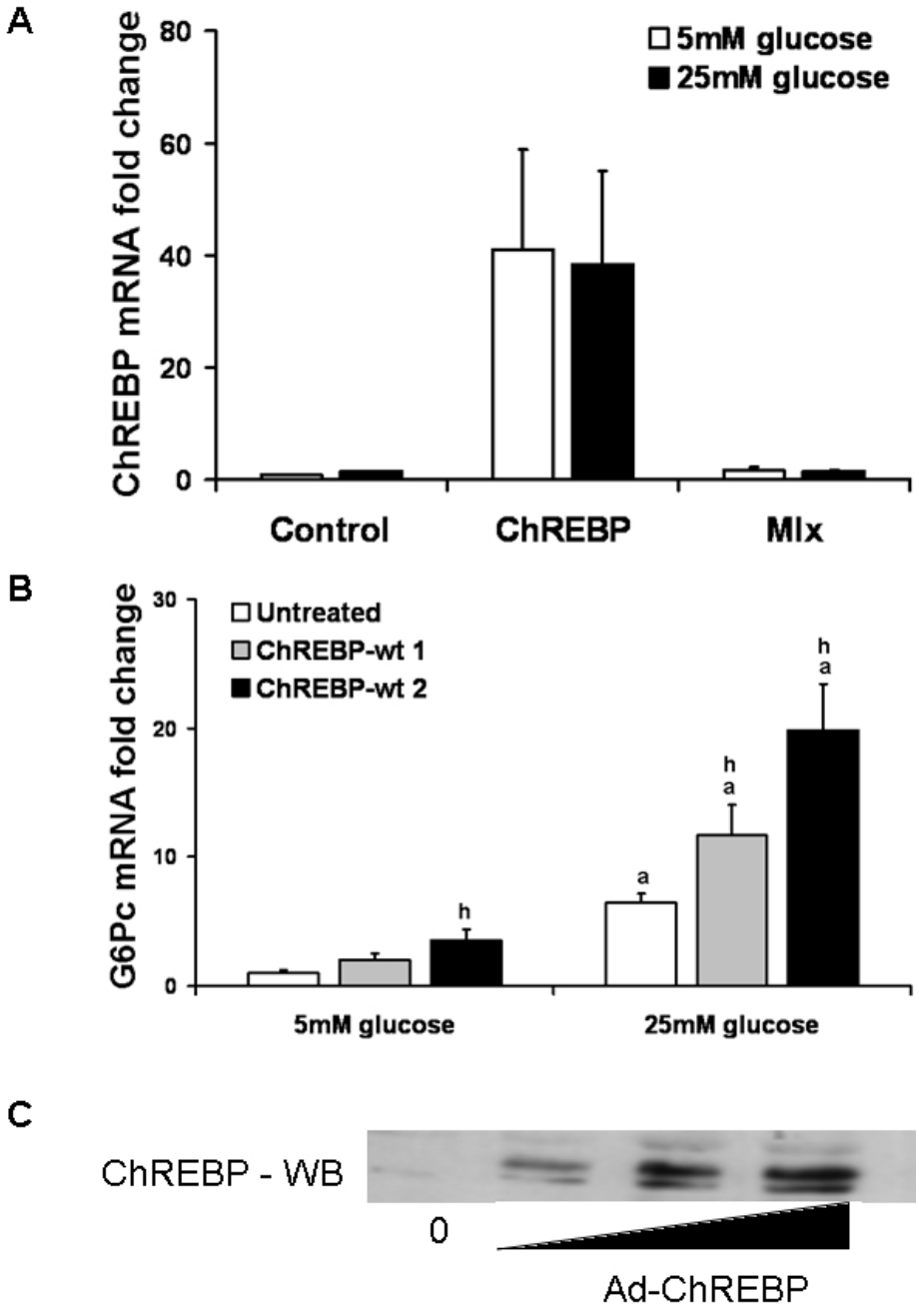


Figure 4.9 Effects of overexpression of ChREBP-WT on G6Pc mRNA expression

Hepatocyte monolayers were either untreated or treated with vectors for expression of ChREBP-WT at two viral titres (two fold dilution). After 18 h pre-culture, they were then incubated for 4 h in MEM containing 5 and 25 mM glucose, indicated for determination of ChREBP (A) and G6Pc (B) mRNA levels and protein expression by immunoblotting assay (C). Mean ± SEM 4 experiments, duplicate treatments (n=8), $^{a}P< 0.05$ effect of glucose, $^{h}P< 0.05$ effect of vectors for expression of ChREBP-WT.

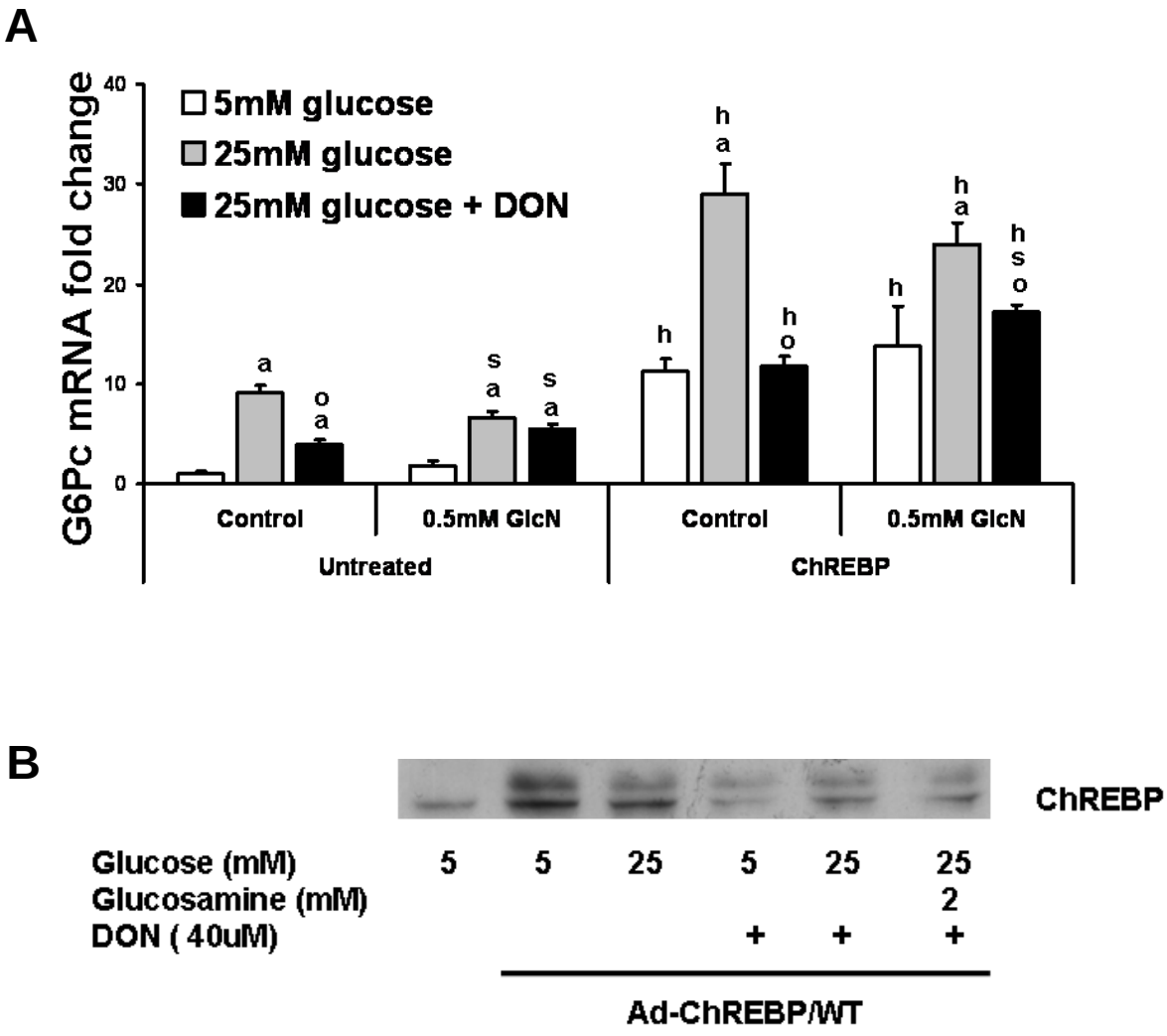


Figure 4.10 Effects of ChREBP on G6Pc mRNA expression

Hepatocyte monolayers were untreated or treated with vectors for expression of ChREBP-WT and pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM glucose, 25 mM glucose and 0.5 mM glucosamine, with 40 µM DON and determination of G6Pc mRNA levels (A) and ChREBP protein expression by immunoblotting assay (B). Mean ± SEM 4 experiments, duplicate treatments (n=8), $^{a}P< 0.05$ effect of glucose, $^{o}P< 0.05$ effect of DON, $^{s}P< 0.05$ effect of GlcN and $^{h}P< 0.05$ effect of ChREBP

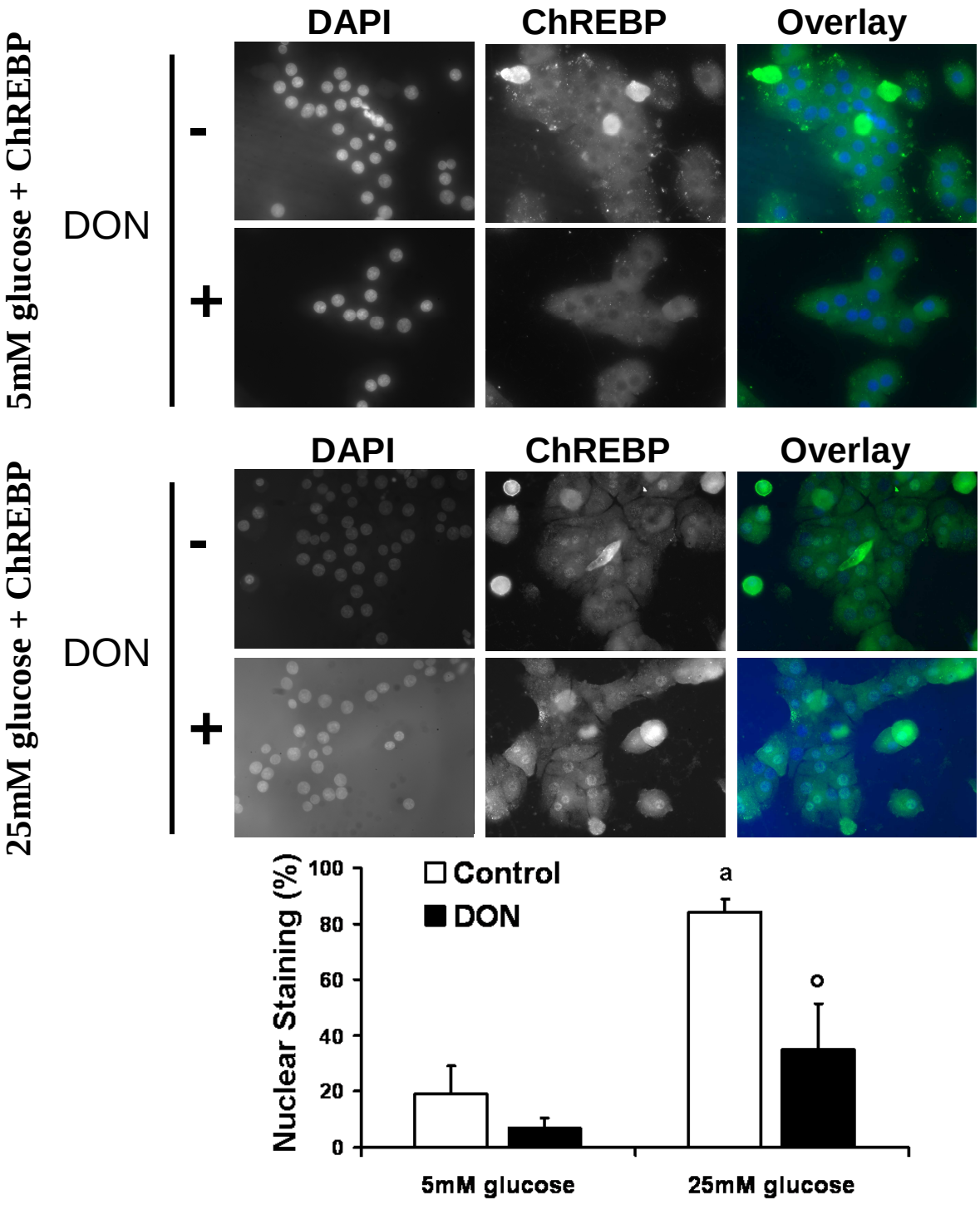


Figure 4.11 Effects of DON on translocation of ChREBP

Hepatocyte were plating and cultured for overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and -/+ DON. They were then incubated for 1 h in MEM containing 5 mM and 25 mM glucose. Cells were scored as having greater levels of ChREBP in the cytoplasm or nucleus as described in methods. Images representative of 2 experiments, coverslips treated in duplicate (n=4). $^{a}P< 0.05$ effect of glucose and $^{o}P< 0.05$ effect of DON.

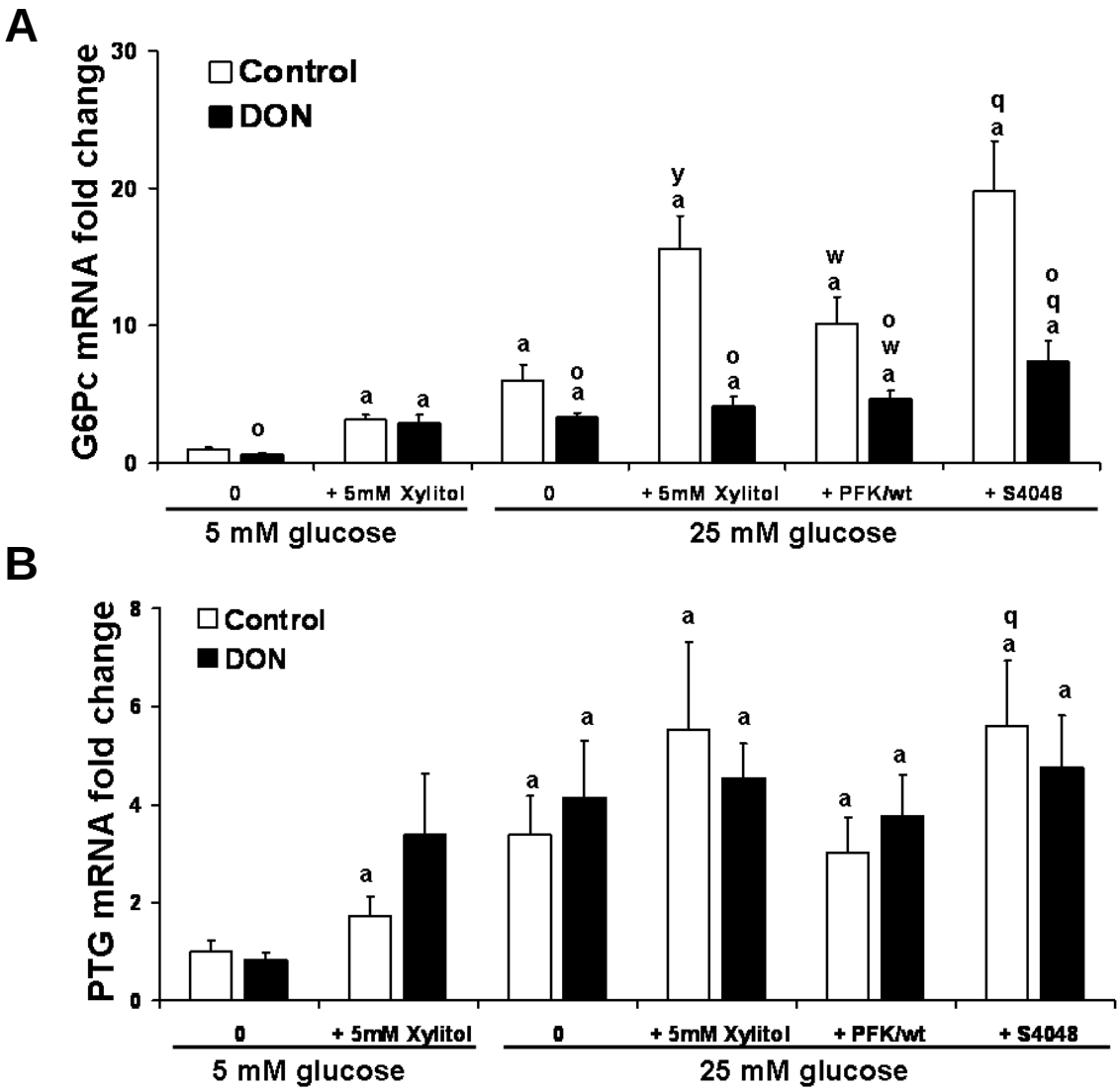


Figure 4.12 Effects of xylitol, PFK-WT and S4048 on gene expression

Hepatocyte monolayers were untreated or treated with vectors for expression of PFK/WT and pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 5 mM xylitol, with 40 µM DON and indicated for determination of gene expression of G6Pc (A) and PTG (B). Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12), $^{a}P< 0.05$ effect of glucose, $^{o}P< 0.05$ effect of DON $^{y}P< 0.05$ effect of xylitol $^{w}P< 0.05$ effect of PFK-WT and $^{q}P< 0.05$ effect of S4048.

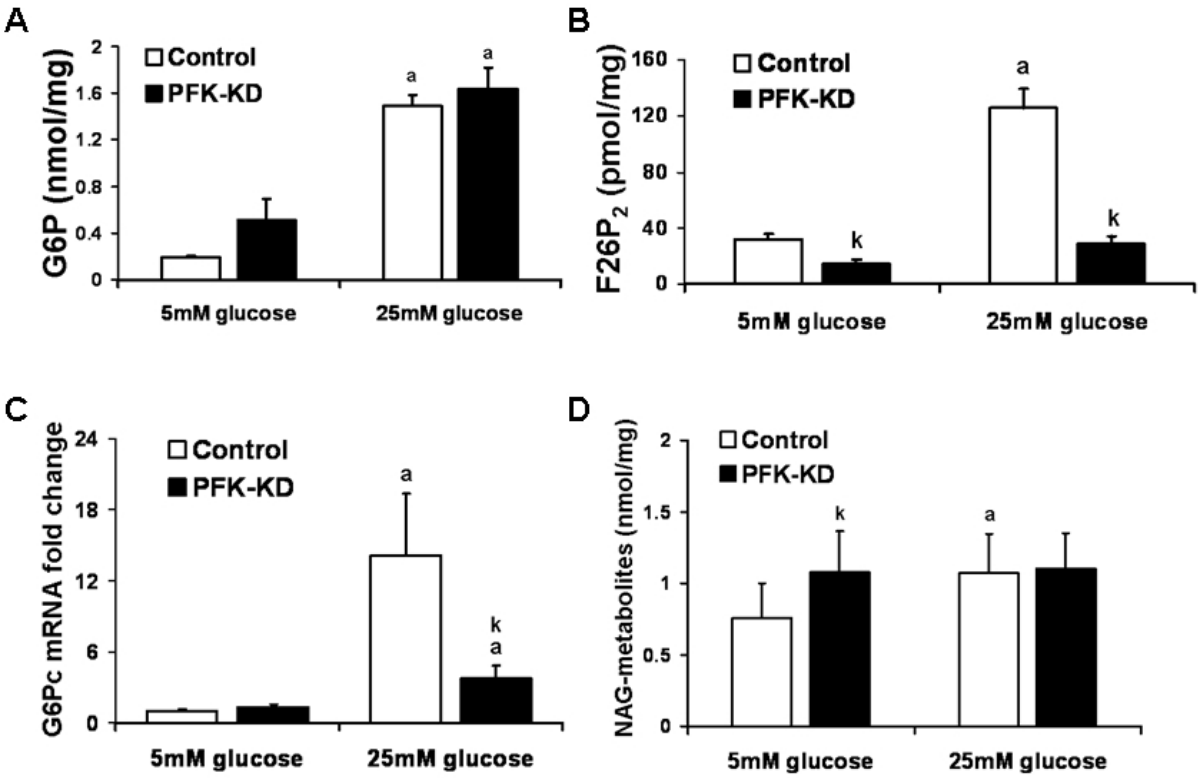


Figure 4.13 Effects of kinase-deficient PFK2/FBP2 (PFK-KD) on G6P, NAG-metabolites, gene expressions and fructose 2,6-bisphosphate

Hepatocyte monolayers were pre-cultured untreated or pre-treated with adenoviral vectors for PFK-KD and overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and indicated for determination of G6P (A), frucotose 2,6-bisphosphate (B), G6Pc mRNA gene expression (C) and NAG-metabolites (D) and Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12), $^{a}P< 0.05$ effect of glucose and $^{k}P< 0.05$ effect of PFK-KD.

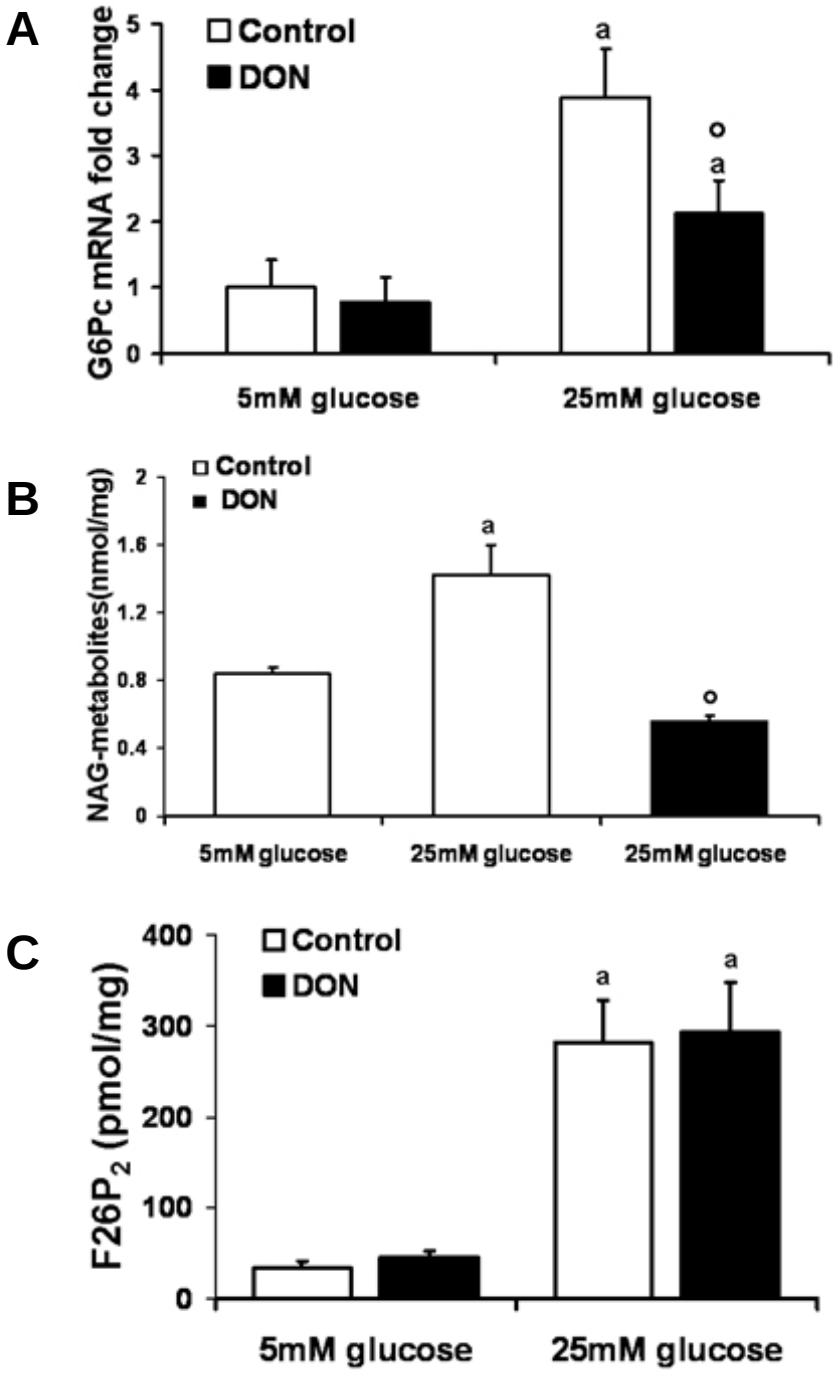


Figure 4.14 Effects of 6-diazo-5-oxonorleucine (DON) on NAG-metabolites, gene expressions and fructose 2,6-bisphosphate

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose, with 40 µM DON and indicated for determination of G6Pc mRNA gene expression (A) NAG-metabolites (B) and frucotose 2,6-bisphosphate (C) Mean ± SEM 4-6 experiments, duplicate treatments (n=8-12), $^{a}P< 0.05$ effect of glucose and $^{o}P< 0.05$ effect of DON.

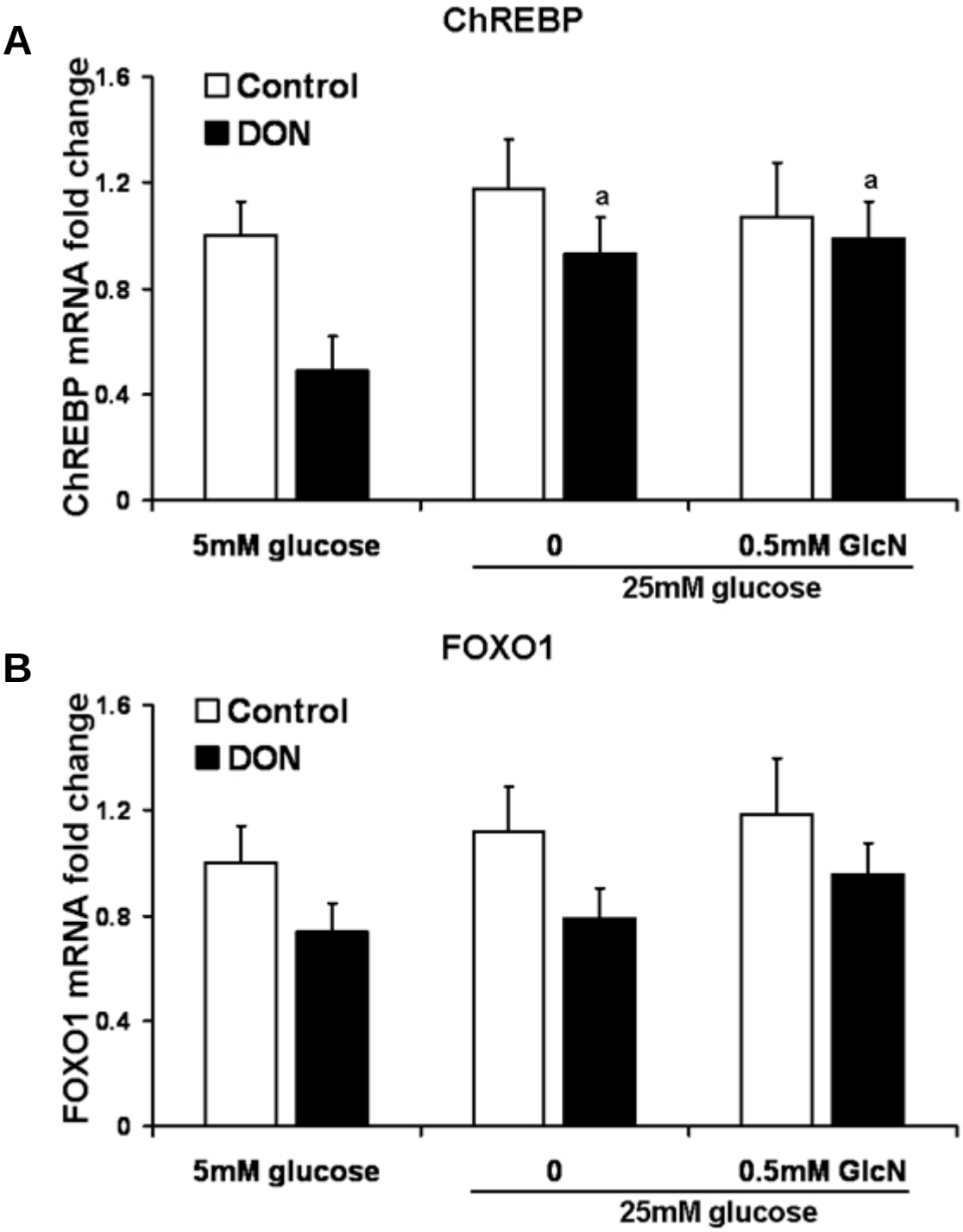


Figure 4.15 Effects of 6-diazo-5-oxonorleucine (DON) and glucosamine (GlcN) on gene expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 0.5 mM glucosamine (GlcN), with 40 µM DON and indicated for determination of gene expression of (A)ChREBP and (B) FOXO1. Mean ± SEM 4 experiments, duplicate treatments (n=8), $^{a}P<0.05$ effect of glucose

Chapter 5

Glucose-induction of G6Pc and TXNIP by Mlx-independent mechanisms

Glucose-induction of G6Pc and TXNIP by Mlx-independent mechanisms

5.1 Aims and rationale

The work in the previous chapter confirmed effects of glucose on G6Pc gene expression in both the absence and the presence of insulin, and it showed that inhibition of the HBP with DON inhibits glucose-induced G6Pc expression and also the induction of G6Pc mRNA caused by overexpression of ChREBP. Although ChREBP is thought to be the major transcription factor that is involved in the regulation of gene expression by high glucose concentration (Ma et al., 2006) other transcription factors may also be involved. Transcription factors involved in regulation of G6Pc expression by insulin include FOXO1 and FOXO3A (Onuma et al., 2006). These transcription factors are targets for covalent modification by O-GlcNAc (Housley et al., 2008; Kuo et al., 2008). The FOXO transcription factors are also regulators of the TXNIP gene in other cell types (Al-Mubarak et al., 2009; de Candia et al., 2008). This gene like G6Pc is induced by glucose and repressed by insulin (Parikh et al., 2007). However, its regulation in liver has been less extensively studied than for G6Pc. In non-hepatic cells including muscle cells and islet beta cells TXNIP is induced by 2-deoxyglucose (2-DOG) and by glucose (Peterson and Ayer, 2011).

The aims of this study were: first to determine the role of Mlx-dependent mechanisms in the glucose induction of G6Pc and TXNIP; second to determine whether these two genes share similar regulatory mechanisms by glucose; third to determine the potential role of FOXO transcription factors in the regulation of these genes by glucose and by the HBP.

5.2 Results

5.2.1 Role of Mlx-dependent mechanisms in the glucose induction of G6Pc and TXNIP

To identify candidate transcription factors involved in the glucose induction of G6Pc and TXNIP we first tested the effects of a dominant-negative variant of Mlx. Mlx forms complexes with ChREBP and MondoA as well as other transcriptional regulators that are thought to function predominantly as repressors including Mad1, Mad4 and Mnt (Billin et al., 1999; 2000; Meroni et al., 2000). Previous studies using the same dominant-negative variant of Mlx showed that during glucose stimulation of gene expression in the presence of insulin, Mlx dependent mechanisms account for a high proportion of gene expression (Ma et al., 2006). The first experiments tested the Mlx-dependence of the glucose induction in both the absence and the presence of insulin. Stimulation of L-PK by glucose was greater in the presence of insulin, whereas stimulation of G6Pc and TXNIP was greater in the absence of insulin (Fig. 5.1, A-C). Overexpression of Mlx-dominant negative (Mlx-DN) abolished the glucose induction of L-PK and also TXNIP and it also abolished the glucose induction of G6Pc in both the absence and presence of insulin. These results together suggest a major role for Mlx-dependent mechanisms in the glucose induction of L-PK, G6Pc and TXNIP in both the absence and the presence of insulin.

5.2.2 Mlx is required for the induction of TXNIP by glucose but not by 2-DOG

Previous studies have shown that TXNIP is induced in various cell types including islet beta cells and other non-hepatic cell lines by 2-DOG (Kaadige et al., 2009; Stoltzman et al., 2011; Minn et al., 2006). However, whether glucose and 2-DOG act through a similar mechanism as is generally assumed remains unclear (Peterson et al., 2011). We therefore tested the effects of overexpression of Mlx-DN on the induction of G6Pc and TXNIP by glucose and 2-DOG (Fig 5.2, A and B). Treatment with Mlx-DN inhibited the glucose induction of TXNIP but significantly increased the induction of TXNIP by 2-DOG. Similar results were obtained when the effects of Mlx-DN were tested over a range of titres (Fig 5.2, C and D). Thus the converse effects of Mlx-DN on the glucose and 2-DOG induction show that these substrates act through different mechanisms.

5.2.3 Expression of FOXO3A causes a greater enhancement of the glucose induction than the 2-DOG induction

Both FOXO1 and FOXO3A are known to be involved in the induction of G6Pc (Guo et al., 1999; Hall et al., 2000) and TXNIP (Fa-Xing and Luo, 2009; Zhuo et al., 2010), and the effects of FOXO1 and FOXO3A on G6Pc are mediated through the same DNA elements (Onuma et al., 2006). We therefore tested the effects of overexpression of FOXO3A on the substrate induction of G6Pc and TXNIP (Fig 5.3, A and B). Overexpression of FOXO3A had a small stimulatory effect on the expression of both G6Pc and TXNIP when measured at 5mM glucose. However, overexpression causes a much larger induction of both G6Pc and TXNIP at 25mM glucose. Interestingly, in incubations with 2-DOG, the effect of FOXO3A overexpression was similar to that in control conditions at 5 mM glucose. When

the effects of a titration of FOXO3A were tested the enhancement at 25 mM glucose was mainly observed at low titres of FOXO3A overexpression (Fig 5.3, C and D). Indicating that unlike the effects of Mlx-DN, the effects of FOXO3A were confined to a narrow range of titres. Low titres (4µl/ml) were therefore used in later studies.

5.2.4 Induction by FOXO3A of the glucose stimulation occurs in both the absence and the presence of insulin

The FOXO transcription factors are major mediators of the insulin repression of G6Pc (Onuma et al., 2006). Additional experiments were performed comparing FOXO3A overexpression in incubations both with and without insulin. In these experiments overexpression of FOXO3A markedly enhanced the stimulation by 25 mM glucose of the induction of G6Pc and TXNIP also in the presence of insulin (Fig 5.4), but had little effect in the presence of 2-DOG.

5.2.5 Mlx-DN inhibits the stimulation by FOXO3A

We next tested whether the induction of G6Pc and TXNIP by FOXO3A is Mlx dependent, by combined overexpression of Mlx-DN and FOXO3A (Fig 5.5). Overexpression of Mlx-DN markedly attenuated the induction by FOXO3A at high glucose of both G6Pc and TXNIP, suggesting that the induction by high glucose is dependent on both FOXO3A and Mlx dependent mechanisms.

5.2.6 Insulin-dependence and glucose counter-regulation of the subcellular location of FOXO1 and FOXO3A

The above studies suggest that the induction of both G6Pc and TXNIP by high glucose in the absence or presence of insulin are dependent on both Mlx and FOXO3A. Because insulin regulates FOXO-dependent mechanisms by Protein kinase B (PKB/AKT) mediated phosphorylation of FOXO proteins which results in the exclusion from the nucleus (Desvergne et al., 2006; Housley et al., 2008), we next tested whether glucose affects the subcellular location of both overexpressed FOXO3A and of endogenous FOXO1. Using commercially available antibodies to FOXO1 we were able to detect endogenous protein. However, with the antibodies to FOXO3A we could only detect the protein when overexpressed with the adenoviral vector.

In cells not treated with insulin, FOXO1 was present in the nucleus of all hepatocytes irrespective of the glucose concentration. Time course studies with insulin showed significant translocation from the nucleus within 15 min during incubation with 5 mM glucose. However translocation in response to insulin measured at 15, 30 and 60 min time points was markedly inhibited by 25 mM glucose (Fig 5.6). The subcellular location of FOXO3A in cells overepressing FOXO3A differed from that of endogenous FOXO1 in that translocation from the nucleus in response to insulin was more rapid for FOXO3A and the effects of 25 mM glucose on increased nuclear staining of FOXO3A were seen in both the absence and the presence of insulin (Fig 5.7). This shows that glucose promotes the retention of both FOXO1 and FOXO3A in the nucleus.

5.2.7 DON inhibits the glucose regulation of FOXO1 and FOXO3A translocation

To test for possible involvement of the hexosamine pathway in the effect of glucose on the subcellular location of FOXO1 and FOXO3A, the effects of DON were determined. Treatment with DON inhibited the effect of high glucose on translocation of both FOXO1 (Fig 5.8) and FOXO3A (Fig 5.9).

5.2.8 DON counteracts O-GlcNAc modification of FOXO3A

We next tested for covalent modification of FOXO3A glycosylation by pull down with wheat germ agglutinin-agarose beads followed by immunoblotting as described previously for ChREBP (Guinez et al., 2011). In Figure 5.10 interestingly, recovery of FOXO3A in the WGA pull-down was greater in the presence of insulin. Pre-treatment with DON markedly counteracted the FOXO3A recovery in the WGA pull-down and combined treatment with GlcN partially reversed the effect of DON. Immunoblotting to FOXO3A protein showed that the effect of DON was on binding to WGA not on total proteins. In parallel experiments DON also inhibited the induction of TXNIP by high glucose in the absence of insulin but had a much smaller effect in the presence of insulin (Fig. 5.11).

5.2.9 OGT overexpression enhances FOXO3A induced G6Pc.

To further test for a possible role for covalent modification of FOXO3A by O-GlcNAc we tested the effects of combined overexpression of FOXO3A and OGT on glucose induced gene expression. Overexpression of OGT was confirmed by

immunoblotting. Overexpression of OGT alone had no significant effect on the glucose induction of G6Pc (Fig 5.12). However, combined overexpression of OGT and FOXO3A modestly enhanced the induction of G6Pc at high glucose, supporting a potential role of covalent modification of FOXO3A in the glucose induction.

5.2.10 Induction of TXNIP by 2-DOG is inhibited by actinomycin-D and calcium antagonist

The above studies indicate involvement of both FOXO transcription factors and Mlx-dependent mechanisms in the induction of G6Pc and TXNIP by high glucose. However, TXNIP but not G6Pc was induced by 2-DOG by an Mlx-independent mechanism (Fig. 5.13). We next tested whether the increase in TXNIP mRNA expression by 2-DOG is due to transcriptional or post-transcriptional mechanisms by incubation with the transcriptional inhibitor actinomycin D. Treatment with actinomycin D totally abolished the increase in mRNA by both 25 mM glucose and 10 mM 2-DOG suggesting that these effects are due to transcriptional rather than post-transcriptional control. Interestingly actinomycin D also markedly lowered TXNIP mRNA levels in basal conditions at 5 mM glucose, suggesting that the mRNA has a short half-life and a high turnover at basal glucose concentration. Immunoblotting to TXNIP protein showed lower immunoactivity in the presence of insulin and also an increase in TXNIP protein with both 25 mM glucose and 2-DOG (Fig. 5.14). We next tested the effects of the calcium channel blocker, verapamil, which was shown in recent studies on islet beta cells and cardiomyocytes to decrease TXNIP mRNA expression in cells treated with 25 mM glucose (Chen et al., 2009; Xu et al., 2012; Cha-Molstad et

al., 2012). Verapamil at a concentration of 150 μM lowered TXNIP mRNA levels by about 70% in basal conditions and also during stimulation with 25 mM glucose or 10 mM 2-DOG. However, unlike actinomycin D which totally abolished the increase in TXNIP mRNA by glucose and 2-DOG, verapamil did not fully block the 4-fold increase by 25 mM glucose or 10 mM 2-DOG (Fig. 5.15). These results suggest that the induction of TXNIP by 2-DOG like that by 25 mM glucose is largely through transcriptional mechanisms. They also suggest that the inhibition of TXNIP expression which had previously been shown only in incubations at high glucose is glucose independent because it does not block the induction by glucose.

5.2.11 Allose and 3-MOG but not 6-DOG, 5TG and glucosamine mimic 2-DOG induction of TXNIP

In various non-hepatic cell lines and also in islet beta cells the induction of TXNIP by 2-DOG is mimicked by 3-O-methylglucose (3-MOG) and allose (Pedersen et al., 2011). We compared the effects of 2-DOG with other glucose analogues and found that similar to non-hepatic cells, TXNIP was induced in hepatocytes by 3-MOG and allose but not by 6-deoxyglucose (6-DOG), which is not a substrate for hexokinase. TXNIP was also not induced by 5-thioglucose (Fig. 5.16). This sugar has been reported to be phosphorylated by hexokinases (Carabaza et al., 1992), but it is also a very potent inhibitor of glucokinase (Agius and Stubbs, 2000). GlcN had no effect on TXNIP mRNA. However, a recent study (Stoltzman et al., 2001) reported that 10 mM glucosamine has a small stimulatory effect on TXNIP expression.

Two other substrates that were found to cause induction of TXNIP mRNA by 3.4-fold and 2.9-fold were fructose (10 mM) which is rapidly metabolised by fructokinase to fructose 1-P and glycerol (2 mM) which is rapidly phosphorylated to glycerol 3-phosphate (Fig. 5.17). Both fructose and glycerol cause acute lowering of ATP. However, none of the glucose analogues caused ATP depletion (results not shown), suggesting that the induction of TXNIP mRNA by the glucose analogues is not due to ATP depletion.

5.2.12 5TG but not bromopyruvate inhibits the induction by the glucose analogs

The induction of TXNIP by glucose analogues in non-hepatic cells has been suggested to be secondary to the phosphorylation of these sugars, based on the inhibition of the glucose effect by 3-bromopyruvate (Stoltzman 2008; Stoltzman et al., 2011) and also the demonstration of phosphate esters of 3-O-methylglucose and allose by mass spectrometry (Stoltzman et al., 2011). Surprisingly, in the present study 3-bromopyruvate, used at the same concentration as in the previous studies (20 μM) had no effect on the induction of TXNIP mRNA by any of the glucose analogues (Fig. 5.18, C). However, 5TG which is a very effective hexokinase inhibitor in primary hepatocytes abolished the induction by 25 mM glucose and significantly inhibited the induction by allose and 3-MOG, but had no significant effect on the induction by 10 mM 2-DOG (Fig. 5.18, A). To investigate the latter further we tested the effects of 3 mM 5TG over a range of concentrations of 2-DOG (2, 5 and 10 mM). In these experiments 5TG significantly attenuated the stimulation by 2 mM 2-DOG but not by higher concentrations (Fig. 5.18B). This suggests that 5TG may be a weak inhibitor of hexokinase with 2-DOG as substrate. The inhibitory effect of 5TG on the

induction of TXNIP by the glucose analogues in hepatocytes suggests a mechanism dependent on the phosphorylation of the analogues, as proposed recently by Stoltzman and colleagues (Stoltzman et al., 2011). Whether the lack of effect of 3-bromopyruvate is due to lack of uptake by hepatocytes or lack of effect on phosphorylation by glucokinase was not tested in this study.

5.2.13 S4048 enhances the induction by glucose but not by 2-DOG

We next tested the effects of the chlorogenic derivative S4048, a potent inhibitor of the glucose 6-P transporter (SLC37A4) that transfers cytoplasmic hexose 6-P into the ER (Härndahl et al., 2006). S4048 causes a large increase in cytoplasmic accumulation of glucose 6-P and also 2-deoxyglucose 6-phosphate (2-DOG 6-P) confirming that both glucose 6-P and 2-DOG 6-P are substrates for SLC37A4 transporter (Arden et al., 2012). SLC37A4 is one of three hexose 6-P transporters present on the ER membrane. A recent study comparing the properties of SLC37A1, SLC37A2 and SLC37A4 in microsomal membranes showed that all three transporters exchange glucose 6-P for Pi but only SLC37A4 is sensitive to chlorogenic inhibition and is coupled to glucose 6-P hydrolysis (Pan et al., 2011). Whether the other transporters are coupled to the hexose 6-phosphate dehydrogenase which has a broader substrate affinity than the cytoplasmic glucose 6-phosphate dehydrogenase is not known (Czegle et al., 2012). Because S4048 inhibits the entry of hexose phosphates into the ER via the SLC37A4 transporter, this means that S4048 limits but does not prevent the entry of glucose 6-P into the ER lumen and it elevates the cytoplasmic concentration of these phosphate esters. We therefore tested the effects of S4048 on the TXNIP induction by the sugars. S4048 significantly increased TXNIP induction by 25

mM glucose but not by 10 mM 2-DOG (Fig. 5.19, A) or by lower 2-DOG concentrations (Fig. 5.19, B). Because S4048 increases 2-DOG 6-P accumulation (Arden et al., 2012), possible explanations for the lack of effect of S4048 on the 2-DOG induction of TXNIP are that it is not mediated by cytoplasmic 2-DOG but by entry of substrate into the ER.

5.2.14 Action of 2-DOG may be mediated by ER stress

Various substrates can be transported into the endoplasmic reticulum (ER), and can induce ER stress at high concentrations, such as glucosamine, tunicamycin and glycosylation inhibitors. Tunicamycin and 2-DOG are inhibitors of N-glycosylation but 2-DOG is less efficient than tunicamycin (Yoshida, 2007; Werstuck et. al., 2006; Kim et. al., 2004). Chaperone proteins such as BiP, GRP78 and GRP94 are induced during endoplasmic reticulum (ER) stress (Sundar Rajan et al., 2007; Robertson et. al., 2006). We therefore tested whether the sugar analogs such as 2-DOG, allose, 3-MOG and 6-DOG induce the ER stress markers GRP78 and GRP94 mRNA expression. In these experiments, we found elevation in both GRP78 and GRP94 by 2-DOG (10 mM) but not by allose (Fig 5.20), which caused a larger increase in TXNIP (Fig. 5.16 and 5.18). Because GRP78 was also induced by 6-DOG which does not induced TXNIP mRNA, these results on GRP78 and GRP94 mRNA do not support a role for ER stress in the induction of TXNIP.

5.2.15 Effects of inhibition of stress kinases and histone deacetylase on TXNIP expression

A recent study suggested that the induction of TXNIP may be mediated by activation of p38 MAPK based on the inhibition of induction by the p38MAPK inhibitor with 4-(4-Fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD1693160) (Li et al., 2009). We tested whether the induction of TXNIP mRNA by glucose or 2-DOG is inhibited by the MAPK inhibitor. Interestingly the induction by glucose but not by 2-DOG was inhibited by PD169316 (Fig. 5.21, A), suggesting that the glucose stimulation but the not that by 2-DOG may involve activation of MAP-kinase. Inhibitors of histone deacetylation (trichostatin A (TSA)) were shown to enhance the induction of TXNIP by glucose in islet beta cells (Cha-Molstad, 2009). We tested whether the effects of 2-DOG are affected by the histone deacetylase inhibitor. The stimulation by glucose was enhanced by TSA, similar to previous findings in beta cells (Fig. 5.21, B). However, the stimulation by 2-DOG was not affected. This further supports a different mechanism for glucose and the glucose analogs.

5.2.16 Glucose and 2-deoxylguocse (2-DOG) Recruitment of Acetyl-Histone 4, NF-Y and FOXO1 to the TXNIP promoter

Previous studies have shown that NF-Y and FOXO binding sites near the glucose response elements in the TXNIP promoter (Yu and Luo, 2009). However, recruitment of NF-Y, Acetyl-Histone 4 (Acetyl-H4), FOXO1 and FOXO3A transcription factors to the TXNIP gene in hepatocytes has not been studied. To test for effects of glucose and 2-DOG on recruitment of transcription factors to the

TXNIP promoter we performed ChIP assays using antibodies to NF-Y, Acetyl-H4, FOXO1 and FOXO3A using primers spanning the two ChoRE sequences. The results showed that binding of acetyl-H4 to the TXNIP promoter was greater with 2-DOG and also binding of FOXO1 was greater with 2-DOG but there was not statistically significant. Binding of NF-Y was less with both 25 mM glucose and 2-DOG but also not statistically significant. There was very little or no binding of FOXO3A with 25 mM glucose (Fig. 5.22).

5.2.17 2-DOG mimics the effect of glucose on FOXO1 and FOXO3A translocation

Comparison of the effect of glucose and 2-DOG on the subcellular location of FOXO1 and FOXO3A (Fig. 5.23) showed that 2-DOG like 25 mM glucose causes increased nuclear staining of both FOXO1 and FOXO3A.

5.3 Discussion

The aims of this study were to test whether glucose induces G6Pc and TXNIP by both Mlx-dependent and Mlx-independent mechanisms and to test the potential role of FOXO transcription factors in regulating the glucose induction of these genes.

Previous studies have shown that TXNIP is induced by glucose in several cell types and repressed by insulin in adipocytes (Parikh et al., 2007). The glucose induction has been shown previously to be mediated by ChREBP-Mlx in islet beta cells (Cha-Molstad et al., 2009) and by MondoA-Mlx in other cell types (Stoltzman et al., 2011). Studies on the TXNIP promoter have identified 2 ChOREs and a FOXO binding site (Yu and Luo, 2009) suggesting that FOXO may have a role in the regulation of this gene. A role for FOXO transcription factors in mediating the activation of TXNIP by cell stress was shown in HepG2/C3A human liver cells and senesant fibroblasts (de Candia et al., 2008; Zhuo et al., 2010). FOXO1 and FOXO3A transcription factors have a major role in the regulation of G6Pc gene expression by insulin (Onuma et al., 2006). However, the role of these regulators in gene regulation by glucose has been less extensively studied.

5.3.1 The role of Mlx-dependent and Mlx-independent mechanisms in glucose regulation

This study shows that the glucose induction of both G6Pc and TXNIP is at least in part Mlx-dependent in both the absence and in the presence of insulin. The induction of G6Pc by glucose is much greater in the absence of insulin than in the presence of insulin and based on titration experiments with different adenoviral

titres of the Mlx-DN it can be concluded that in the absence of insulin there may also be Mlx-independent mechanism. However, in the presence of insulin there was no clear evidence for an Mlx-independent stimulation of G6Pc. Because insulin causes a large inhibition of G6Pc expression and glucose causes a large stimulation in the absence of insulin; the simplest explanation is that Mlx-independent stimulation of G6Pc by glucose is inhibited by insulin and the Mlx-dependent stimulation is not inhibited by insulin.

The regulation of TXNIP by glucose and insulin differs from G6Pc regulation in two ways: (i) The stimulation by glucose (in the absence of insulin) is smaller for TXNIP. (ii) The inhibition by insulin is also smaller for TXNIP than for G6Pc. Also the experiments with the glucose analogues show clearly that this mechanism is unique to TXNIP but does not apply to G6Pc and also that this glucose analogue stimulation is by an Mlx-independent mechanism. However, as discussed below, the glucose analogues may be acting by a different mechanism from glucose. This study provides evidence that glucose induces both G6Pc and TXNIP more strongly in the absence of insulin than in the presence of insulin. Because the FOXO transcription factors are major negative regulators of insulin action, they are strong candidates for the stimulation by glucose of these genes.

5.3.2 The role of FOXO transcription factors in glucose regulation

Endogenous expression of FOXO1 was clearly detectable by immunostaining in hepatocytes and it was present in the nucleus in hepatocytes not incubated with insulin and it translocated to the cytoplasm within 15 to 60 min of addition of insulin to the hepatocytes. Endogenous expression of FOXO3A was not detectable in hepatocytes suggesting that FOXO3A may be expressed at lower

levels than FOXO1. When FOXO3A was overexpressed with low adenoviral titres it was clearly present in the nucleus at much higher concentrations than in the cytoplasm, although it was also present in the cytoplasm. Interestingly, FOXO3A translocated from the nucleus in response to insulin much more rapidly than FOXO1. The effects of overexpression of FOXO3A on G6Pc and TXNIP gene expression were tested over a range of concentrations in the titration experiments, and both genes were induced by FOXO3A overexpression. However, the effect was bisphasic and was greatest at the low titres of FOXO3A. Overexpression of FOXO3A stimulated both G6Pc and TXNIP at 5 mM glucose but caused a much greater stimulation of both these genes at 25 mM glucose. For both genes this stimulation was greatest at the lowest titres of FOXO3A expression. The immunostaining experiments showed that glucose caused the accumulation of FOXO1 and FOXO3A in the nucleus in the absence of insulin as expected from studies on other cell types (Wilk et al., 2011; Chong et al., 2011; Meur et al., 2011; Cifarelli et al., 2012) and also in the presence of insulin. The effect of glucose on nuclear accumulation of FOXO3A was much smaller in the presence of insulin, because of the very rapid and stronger effect of insulin on nuclear exclusion of FOXO3A compared with FOXO1. Together these results suggest that the FOXO transcription factors are very strong candidates for the glucose stimulation of both G6Pc and TXNIP especially in the absence of insulin.

Experiments testing combined expression of FOXO3A and Mlx-DN suggested that the induction of both genes by FOXO3A may also be at least in part Mlx-dependent. This suggests that there may be an interaction between Mlx-mechanisms and FOXO-dependent stimulation. There may also be other transcription factors involved in the Mlx-independent stimulation of G6Pc and TXNIP by high glucose.

5.3.3 Regulation of FOXO transcription factors by glucose and the HBP

This study shows that the GFAT inhibitor DON strongly inhibits the glucose-induced expression of both G6pc and TXNIP in the absence of insulin but has a smaller effect in the presence of insulin. The FOXO transcription factors are covalently modified on various serine / threonine residues by PKB/Akt which is activated by insulin (Gross et al., 2009) and they are also covalently modified by O-GlcNAc (Housley et al., 2009). Insulin and 25 mM glucose had opposite effects on the subcellular location of both FOXO1 and FOXO3A. Insulin caused the movement of FOXO1 and FOXO3A from the nucleus to the cytoplasm and 25 mM glucose opposed the effect of insulin by causing the accumulation of both FOXO1 and FOXO3A in the nucleus in the presence of insulin. This "counter-regulatory" effect of high glucose on the insulin translocation out of the nucleus could be due to either inhibition of insulin signalling or to an effect of glucose that is independent of insulin signalling. Other studies from our lab showed that high glucose does not affect the stimulation by insulin of Akt phosphorylation (Arden et al., 2011) suggesting that high glucose may be acting by other mechanisms. In this study the GFAT inhibitor DON, opposed the effect of high glucose on the sub-cellular location of both FOXO1 and FOXO3A. The effects of DON were greater on FOXO3A than on FOXO1. This inhibitory effect of DON on the glucose induced retention of the FOXO transcription factors in the nucleus suggests involvement of the HBP and O-GlcNAc modification of FOXO transcription factors by high glucose resulting in their retention in the nucleus and also involvement of these FOXO regulators in the glucose induction of G6Pc and TXNIP. Pull-down experiments of O-GlcNAc modified proteins with wheat-germ agglutinin in hepatocytes overexpressing FOXO3A confirmed that incubation

with high glucose increases the covalent modification of FOXO3A by O-GlcNAc and that this effect is reversed by DON and also by insulin. The stimulation of G6Pc expression by combined expression of OGT and FOXO3A also supports a role for modification of FOXO3A by O-GlcNAc in the glucose induction of G6Pc. Together these results support a model that the glucose induction of both G6pc and TXNIP involves the covalent modification of FOXO transcription factors by O-GlcNAc causing their retention in the nucleus, and that the inhibition by the GFAT inhibitor involves decreased covalent modification of FOXO transcription factors and decreased retention in the nucleus.

5.3.4 Effects of glucose analogues on TXNIP expression

A major difference between G6Pc and TXNIP regulation is that TXNIP but not G6Pc was induced by glucose analogues. Studies on adipocytes, muscle cells and other non-liver cell types have shown inhibition of TXNIP expression by insulin and induction by glucose (Parikh et al., 2007), 2-DOG and other glucose analogues including 3-MOG (Stoltzman et al., 2011). However, whether glucose and 2-DOG act by the same or different mechanisms was not demonstrated (Stoltzman et al., 2011).

Several experiments suggested that the induction of TXNIP by 2-DOG involves a different mechanism from the regulation by glucose: (i) induction by 2-DOG was Mlx-independent; (ii) unlike induction by glucose it was not enhanced by the histone deactylase inhibitor, trichostatin A; (iii) unlike the induction by glucose it was not inhibited by the MAP-kinase inhibitor. The effect of 2-DOG is most likely due to control of gene transcription because it was inhibited by the transcription inhibitor, actinomycin D. A previous study by Petersen and Ayer

suggested that 2-DOG needs to be phosphorylated to induce TXNIP expression (Petersen and Ayer, 2011). Our results are consistent with a phosphorylation mechanism because 6-DOG which cannot be phosphorylated and 5TG which is only weakly phosphorylated did not induce TXNIP expression. Also, 5TG, which is a hexokinase inhibitor, inhibited the induction by allose and partially inhibited the induction by 3-MOG and low concentrations of 2-DOG, consistent with a mechanism that involves phosphorylation of the analogues. After phosphorylation of 2-DOG to 2-DOG 6-P it is not further metabolised by glycolysis. However, it is metabolised by glucose 6-P because the chlorogenic derivative which inhibits transport hexose 6-phosphates into the ER causes accumulation of both glucose 6-P and 2-DOG 6-P (Arden et al., 2012). The action of the glucose analogues on TXNIP expression could occur by a mechanism in either the cytoplasm or the ER. Unlike the induction of TXNIP by glucose, the induction by 2-DOG was not enhanced by the chlorogenic derivative (S4048) which inhibits the glucose 6-P transporter on the ER membrane that is coupled to glucose 6-P (Pan et al., 2011). A possible explanation for the lack of effect of S4048 on the 2-DOG stimulation of TXNIP is that the glucose analogues may be phosphorylated in the cytoplasm and transported into the ER on other transporters that are insensitive to S4048 (Pan et al., 2011) and metabolism of the analogues in the ER lumen by the hexose 6-phosphate dehydrogenase may be involved in TXNIP regulation. Further work using inhibitors of the hexose 6-phosphate dehydrogenase would be required to test this possibility (Zielinska et al., 2010). The induction of the ER stress markers GRP78 and GRP94 is consistent with a possible role of metabolism in the ER in the induction of TXNIP. Two sets of evidence support possible involvement of FOXO transcription factors in the regulation of TXNIP by the glucose analogues. First, overexpression of FOXO3A at low concentrations enhanced the induction

of TXNIP by 2-DOG. Second, 2-DOG and 3-MOG increased the nuclear accumulation of both FOXO1 and FOXO3A. Similar results were not observed for allose which is a strong inducer of TXNIP. However in some experiments the combination of FOXO3A overexpression and treatment with allose resulted in cell death (results not shown). Measurement of mRNA changes can only be performed on the whole cell population, whereas nuclear accumulation of FOXO transcription factors is measured in individual cells. We cannot exclude the possibility that the glucose analogs may induce TXNIP in a small proportion of the cells and the formation of TXNIP protein may lead to apoptosis as has been reported for islet beta cells (Minn et al., 2005).

5.3.5 Role of histone acetylation in TXNIP expression

Previous studies (Butler et al., 2002) testing the effects of inhibition of Histone deacetylases (HDAC) with SAHA followed by gene microarrays identified TXNIP as the main gene that was induced 2-fold by inhibition of HDAC. In this study they identified through promoter deletion constructs that the region of the TXNIP promoter contains an NF-Y binding site and they confirmed using a dominant negative NF-Y construct that the induction of TXNIP expression by the HDAC inhibitor is dependent on NF-Y binding. These results suggest that TXNIP expression is regulated by HDAC binding to NF-Y (Butler et al., 2002) and that deacetylation by HDAC inhibits TXNIP expression. Later work confirmed the induction of TXNIP by HDAC inhibitors and showed that combined inhibition of HDAC with TSA and methylation with 5-aza caused a greater induction of TXNIP (Lee et al., 2010). A recent study on islet beta cells showed that the HDAC inhibitor stimulated TXNIP expression at both 5mM and 25mM glucose

and it caused increased binding of Acetyl-H3 and Acetyl-H4 at the TXNIP promoter. They also showed an increase in recruitment of Acetyl-H4 in response to glucose (Cha-Molstad et al., 2009).

Two key findings from the present study are: (i) TSA increased TXNIP expression with 25mM glucose but not with 2-DOG; (ii) Binding of Acetyl-H4 to the TXNIP promoter was significantly increased by 2-DOG but not by 25mM glucose. The simplest explanation for these two observations is that in the presence of 2-DOG there is little or no binding of HDAC to the TXNIP promoter. This would explain both the lack of effect of TSA and the very high Acetyl-H4. Interestingly, Butler et al., (2002) concluded that HDAC binds to the TXNIP promoter through the NF-Y site. In the ChIP experiments in this study there was little or no binding of NF-Y with 25mM glucose and 2-DOG. A possible explanation is that there are other binding sites for HDAC apart from NF-Y that are activated by glucose but not by 2-DOG. One possibility would be an Mlx-dependent mechanism, because this was activated by glucose but not by 2-DOG. In addition the induction of TXNIP by 2-DOG was increased by the Mlx-dominant negative, which also supports this explanation. Therefore the activation of TXNIP by 2-DOG and other analogues is most likely explained by dissociation of HDAC and increased histone acetylation.

5.4 Summary

This study has shown that:

- Mlx-dependent mechanisms are involved in the glucose induction of both G6Pc and TXNIP mRNA expression but not in the induction of TXNIP by 2-DOG.

- A major role for FOXO transcription factors in the converse effects of glucose and insulin on the expression of both G6Pc and TXNIP is supported by: (i) the enhanced expression of these genes at high glucose by FOXO3A overexpression; (ii) the opposite effects of glucose and insulin on the subcellular location of FOXO1 and FOXO3A.

- A role for the HBP in the glucose regulation of G6Pc and TXNIP by FOXO transcription factors is supported by the following sets of evidence: (i) inhibition by DON on glucose induced FOXO translocation; (ii) greater inhibition by DON of glucose induction of these genes in the absence of insulin; (iii) inhibition of covalent modification of FOXO3A by O-GlcNAc as determined in the wheat germ agglutin assays; (iv) additive induction of G6pc in incubations with overexpression of OGT and FOXO3A at high glucose.

- Induction of TXNIP by glucose analogues is mediated by a different mechanism from the glucose induction as supported by: (i) lack of Mlx-dependence; (ii) lack of effect of stress kinase inhibitors and histone deacetylase inhibitors; (iii) increased binding of histone Acetyl-H4 indicating increased histone acetylation.

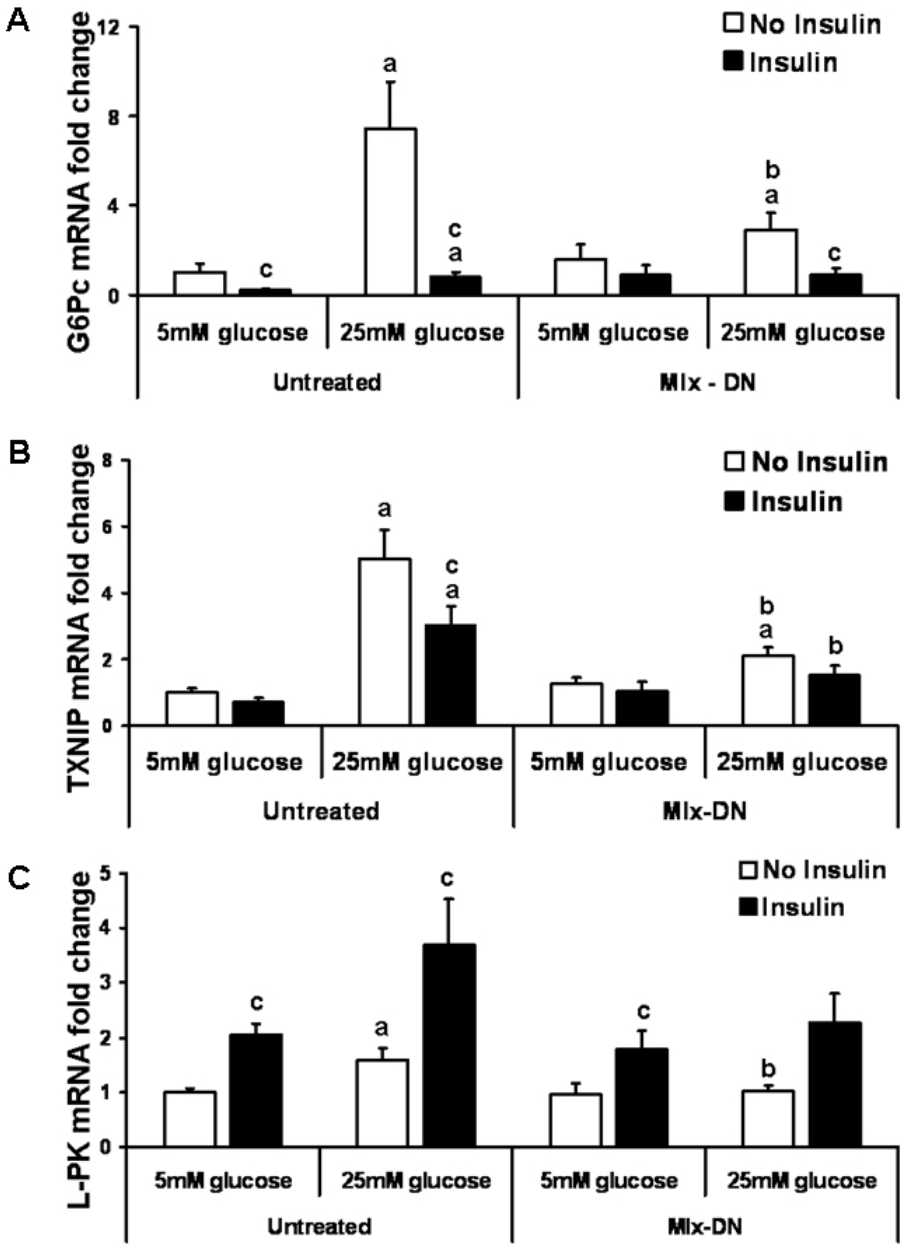


Figure 5.1 Effects of Mlx-DN on G6Pc, TXNIP and L-PK mRNA expression

Hepatocytes were untreated or treated with adenoviral vectors for dominant negative (DN) variant of Mlx Mlx-DN in serum free MEM were added to cells at 2 to 4 h after plating and cultured for 4 h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose, with or without 10nM insulin and indicated for determination of gene expression (A) G6Pc, (B) TXNIP and (C) L-PK. Mean ± SEM 8 experiments, duplicate treatments (n=16) , $^{a}P< 0.05$ effect of glucose, $^{b}P< 0.05$ effect of Mlx-DN, $^{c}P< 0.05$ effect of insulin.

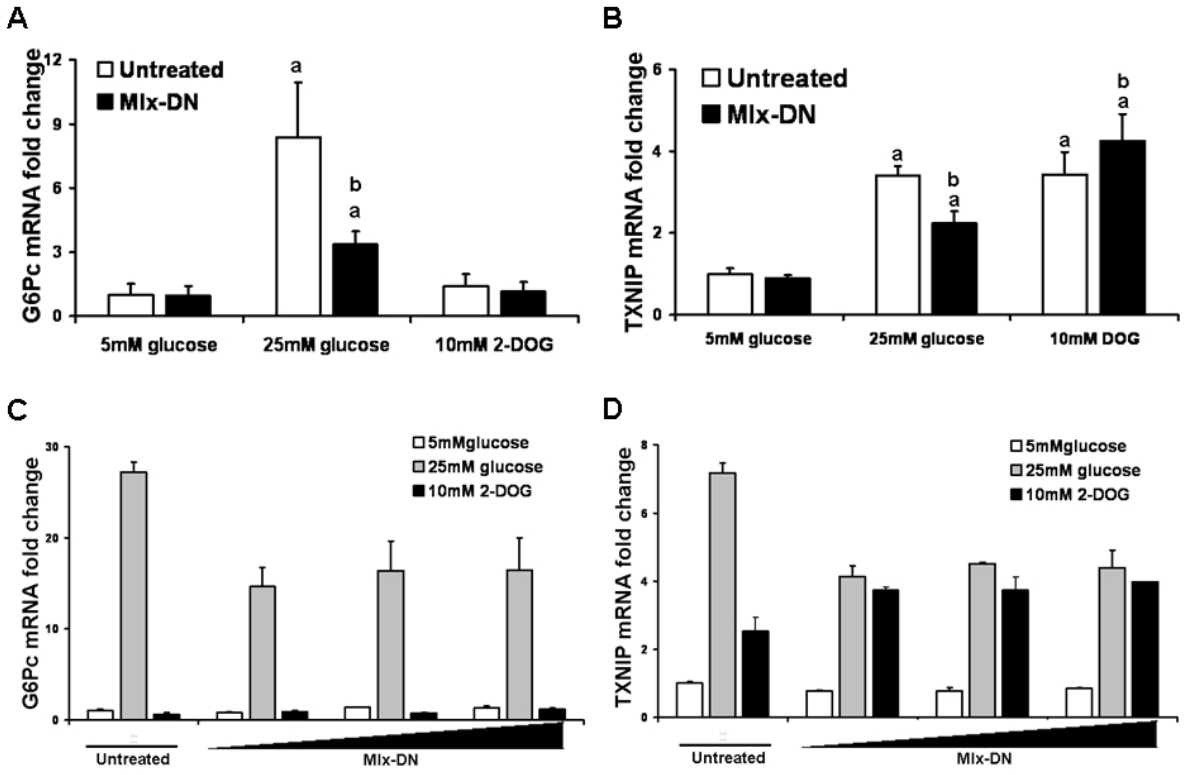


Figure 5.2 Effects of Mlx-DN on G6Pc and TXNIP mRNA expression

Hepatocytes were untreated or treated with adenoviral vectors for Mlx-DN in serum free MEM were added to cells at 2 to 4 h after plating and cultured for 4 h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG) and indicated for determination of gene expression, 6 experiments, duplicate treatments (n=12) (A) G6Pc, (B) TXNIP and overexpression of Mlx-DN, 2 experiments, duplicate treatments (n=4) (C) G6Pc and (D) TXNIP. Mean ± SEM, $^{a}P< 0.05$ effect of glucose, $^{b}P< 0.05$ effect of Mlx-DN.

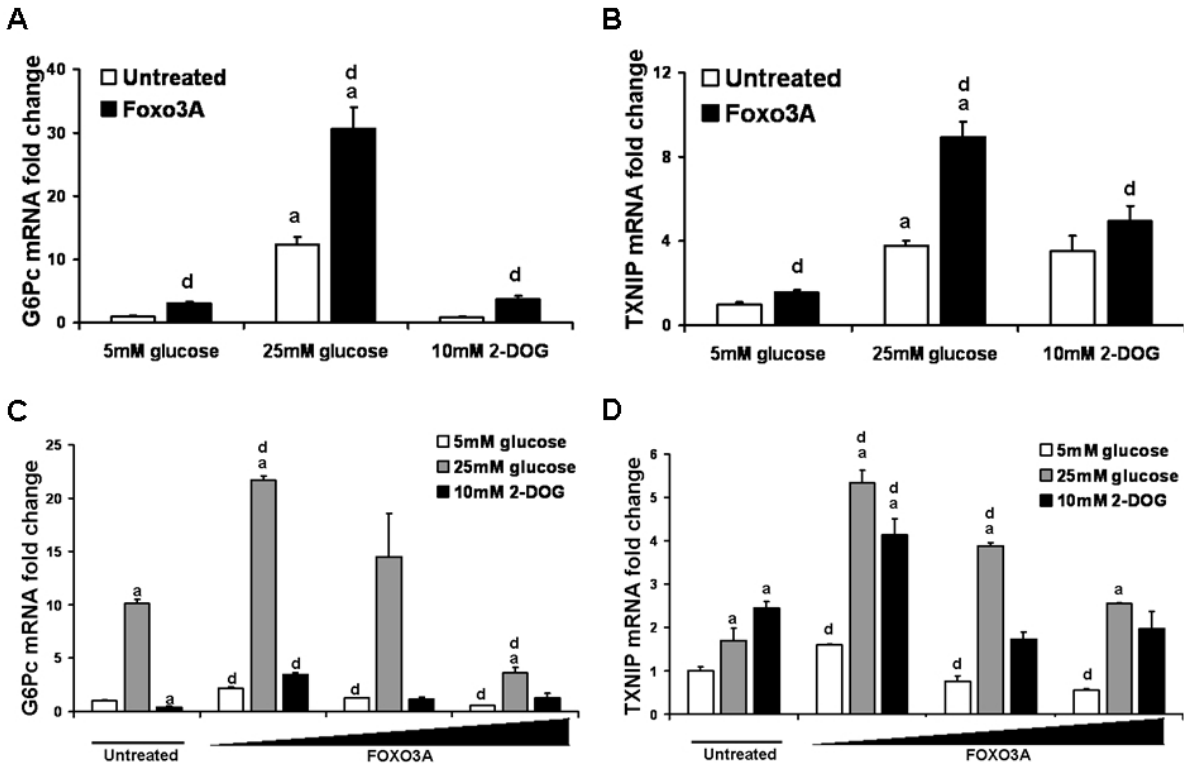


Figure 5.3 Effects of FOXO3A on G6Pc, TXNIP mRNA expression

Hepatocytes were untreated or treated with adenoviral vectors for FOXO3A in serum free MEM were added to cells at 2 to 4 h after plating and cultured for 4 h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG) and indicated for determination of gene expression, 6 experiments, duplicate treatments (n=12) (A) G6Pc, (B) TXNIP and overexpression of FOXO3A, 2 experiments, duplicate treatments (n=4) (C) G6Pc and (D) TXNIP. Mean ± SEM, $^{a}P< 0.05$ effect of glucose, $^{d}P< 0.05$ effect of FOXO3A.

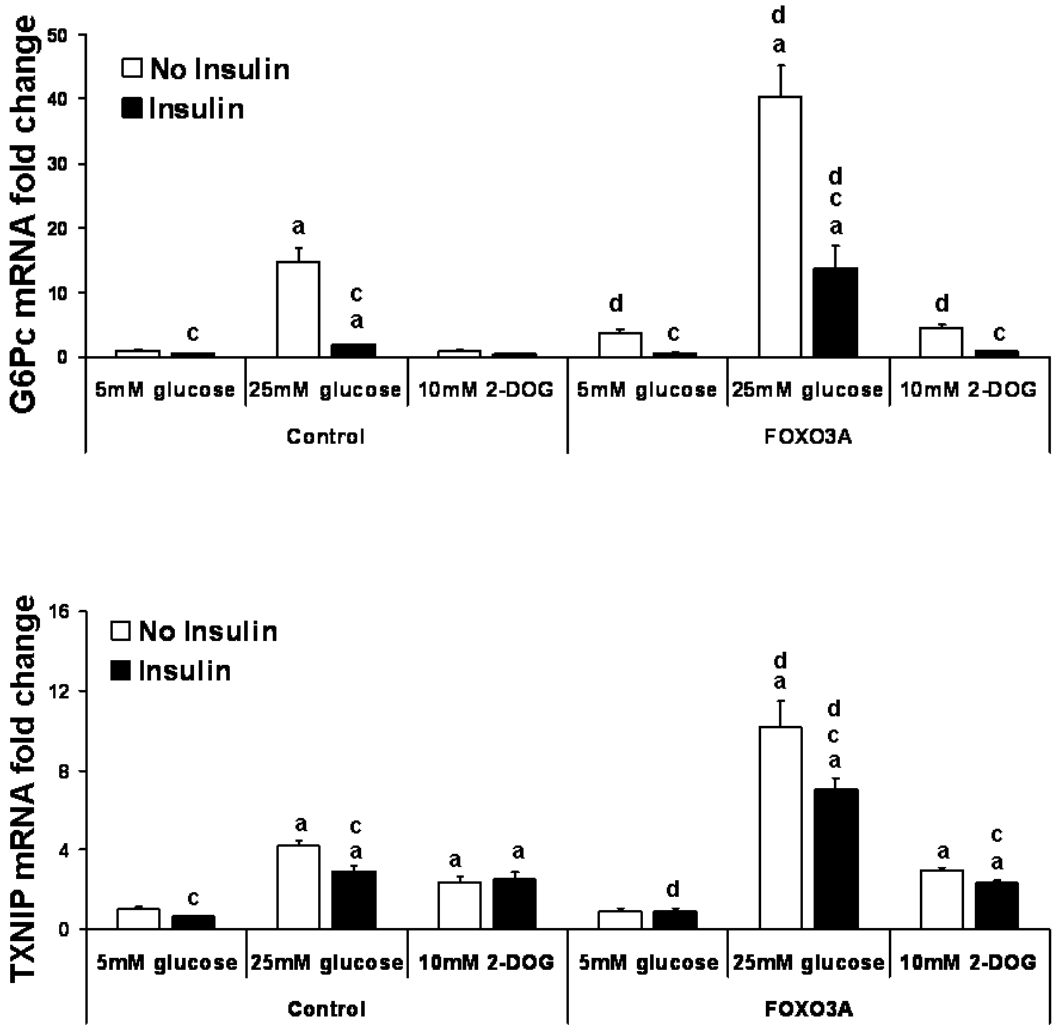


Figure 5.4 Effects of FOXO3A without or with insulin on G6Pc and TXNIP mRNA expression

Hepatocytes were untreated or treated with adenoviral vectors for FOXO3A in serum free MEM were added to cells at 2 to 4 h after plating and cultured for 4 h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG), without or with insulin and indicated for determination of gene expression, G6Pc and TXNIP. Means ± SEM for 4 experiments, duplicate treatments (n=8), $^{a}P< 0.05$ effect of glucose, $^{c}P< 0.05$ effect of insulin and $^{d}P< 0.05$ effect of FOXO3A.

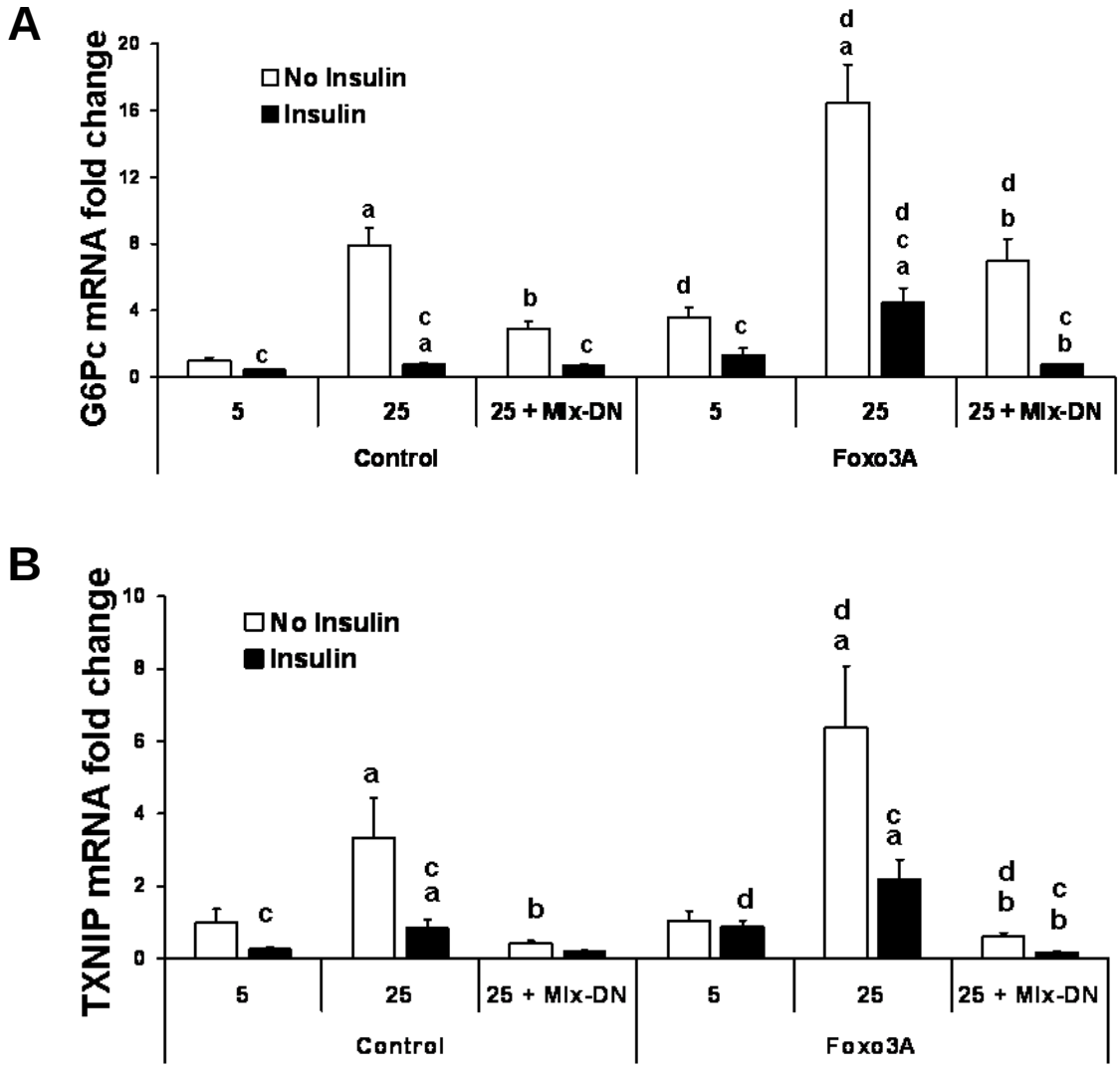


Figure 5.5 Effects of FOXO3A and Mlx-DN on G6Pc and TXNIP mRNA expression

Hepatocytes were untreated or treated with adenoviral vectors for wild type FOXO3A and dominant negative (DN) variant ofMlx in serum free MEM were added to cells at 2 to 4 h after plating and cultured for 4 h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose, with or without 10 nM insulin and indicated for determination of gene expression (A) G6Pc and (B) TXNIP. Mean ± SEM 4 experiments, duplicate treatments (n=8), $^{a}P< 0.05$ effect of glucose, $^{b}P< 0.05$ effect of Mlx-DN, $^{c}P< 0.05$ effect of insulin and $^{d}P< 0.05$ effect of FOXO3A.

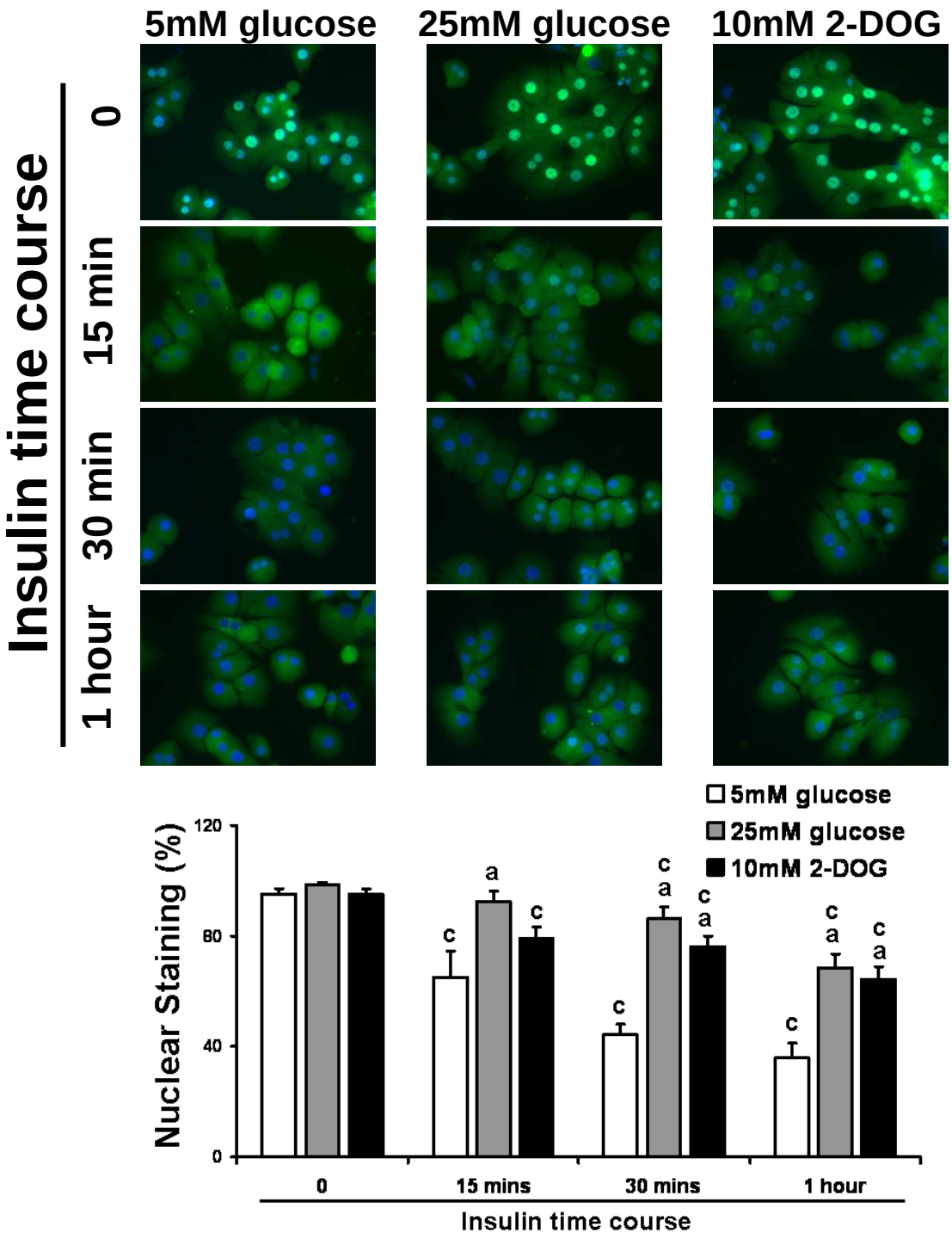


Figure 5.6 Effects of insulin on translocation of FOXO1

Hepatocyte were plating and cultured for overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 1 h in MEM containing 5 mM and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG), with time course of 10 nM insulin. Cells were scored as having greater levels of FOXO1 in the cytoplasm or nucleus as described in methods. Images representative of 2 experiments, coverslips treated in duplicate (n=4). $^{a}P< 0.05$ effect of glucose and $^{c}P< 0.05$ effect of insulin.

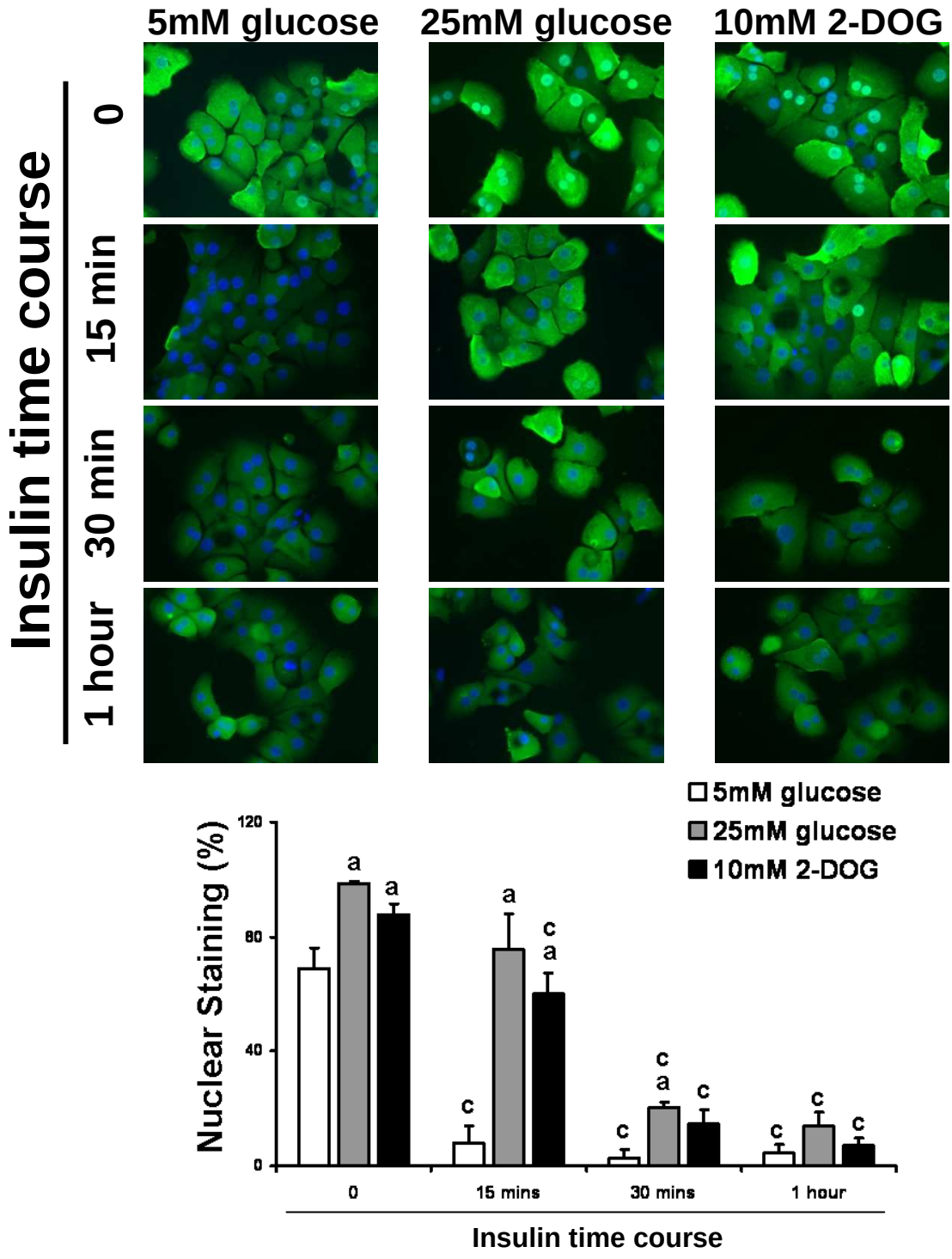


Figure 5.7 Effects of insulin on translocation of FOXO3A

Hepatocyte were plating and cultured for overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 1 h in MEM containing 5 mM and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG), with time course of 10 nM insulin. Cells were scored as having greater levels of FOXO3A in the cytoplasm or nucleus as described in methods. Images representative of 2 experiments, coverslips treated in duplicate (n=4). $^{a}P< 0.05$ effect of glucose and $^{c}P< 0.05$ effect of insulin.

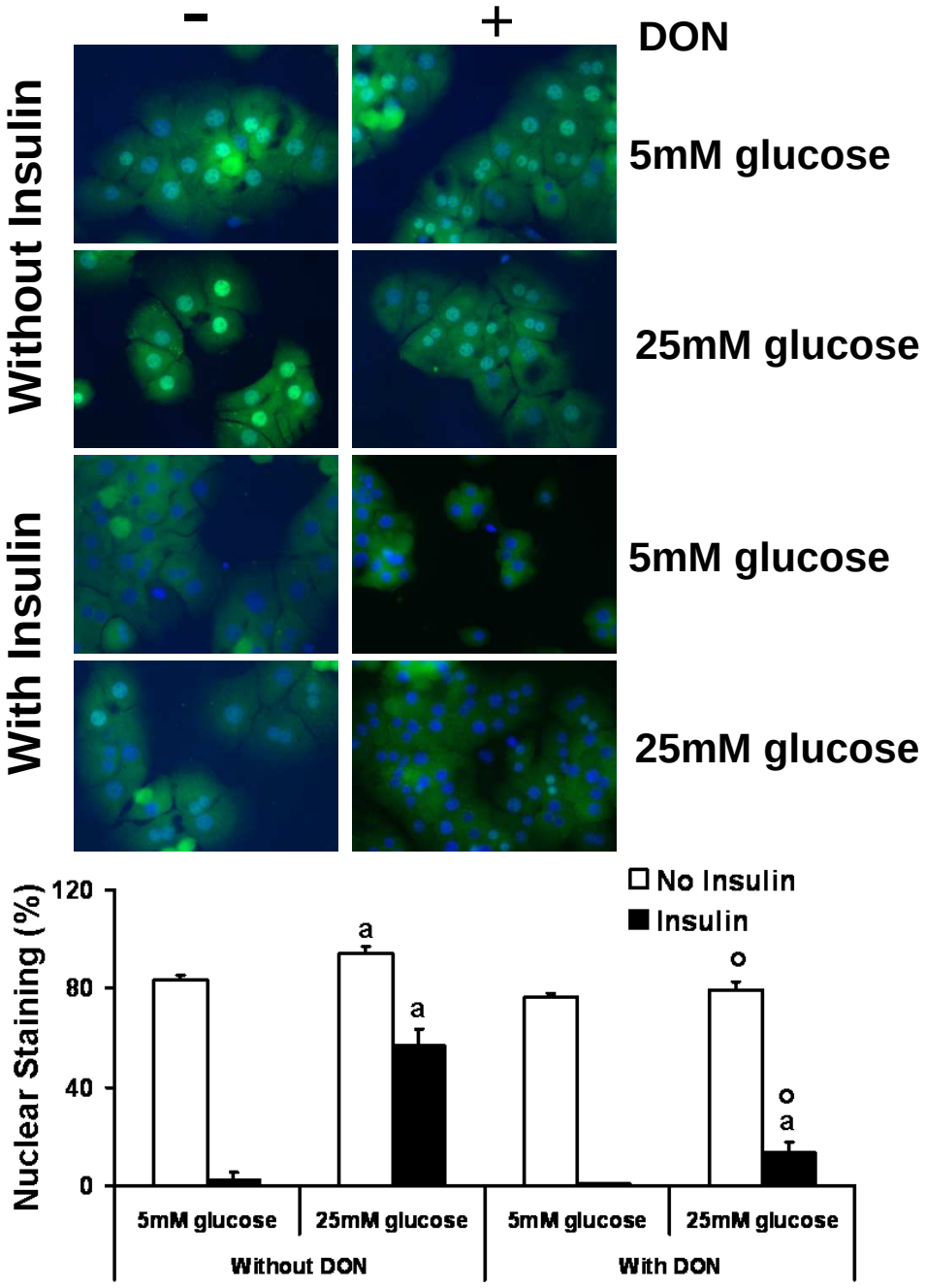


Figure 5.8 Effects of DON on translocation of FOXO1

Hepatocyte were plating and cultured for overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and -/+ DON. They were then incubated for 1 h in MEM containing 5 mM and 25 mM glucose, with or without 10 nM insulin for 15 min. Cells were scored as having greater levels of FOXO1 in the cytoplasm or nucleus as described in methods. Images representative of 2 experiments, coverslips treated in duplicate (n=4). $^{a}P< 0.05$ effect of glucose and $^{o}P< 0.05$ effect of DON.

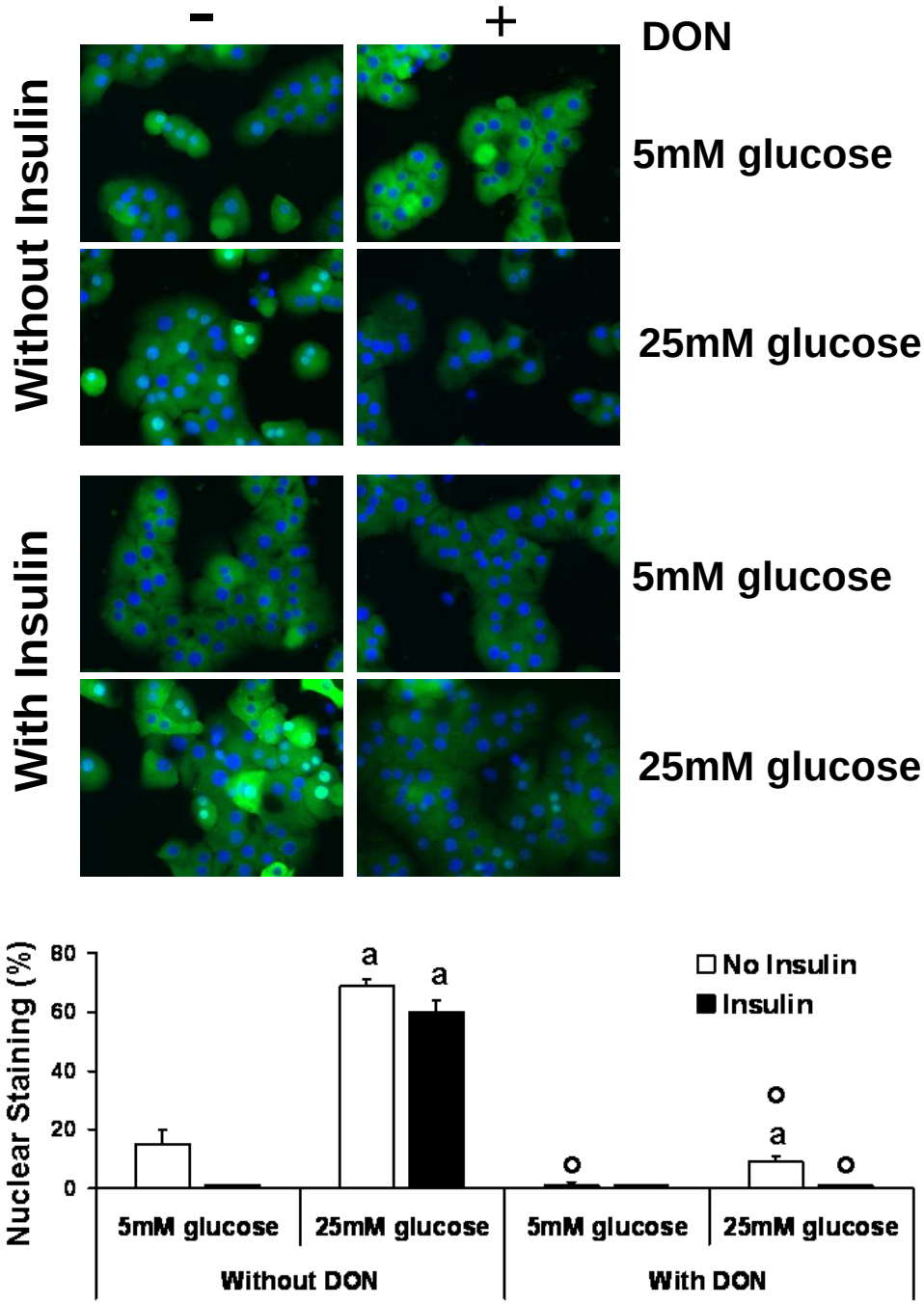


Figure 5.9 Effects of DON on translocation of FOXO3A

Hepatocyte were plating and cultured for overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and -/+ DON. They were then incubated for 1 h in MEM containing 5 mM and 25 mM glucose, with or without 10 nM insulin for 15 min. Cells were scored as having greater levels of FOXO3A in the cytoplasm or nucleus as described in methods. Images representative of 2 experiments, coverslips treated in duplicate (n=4). $^{a}P< 0.05$ effect of glucose and $^{o}P< 0.05$ effect of DON.

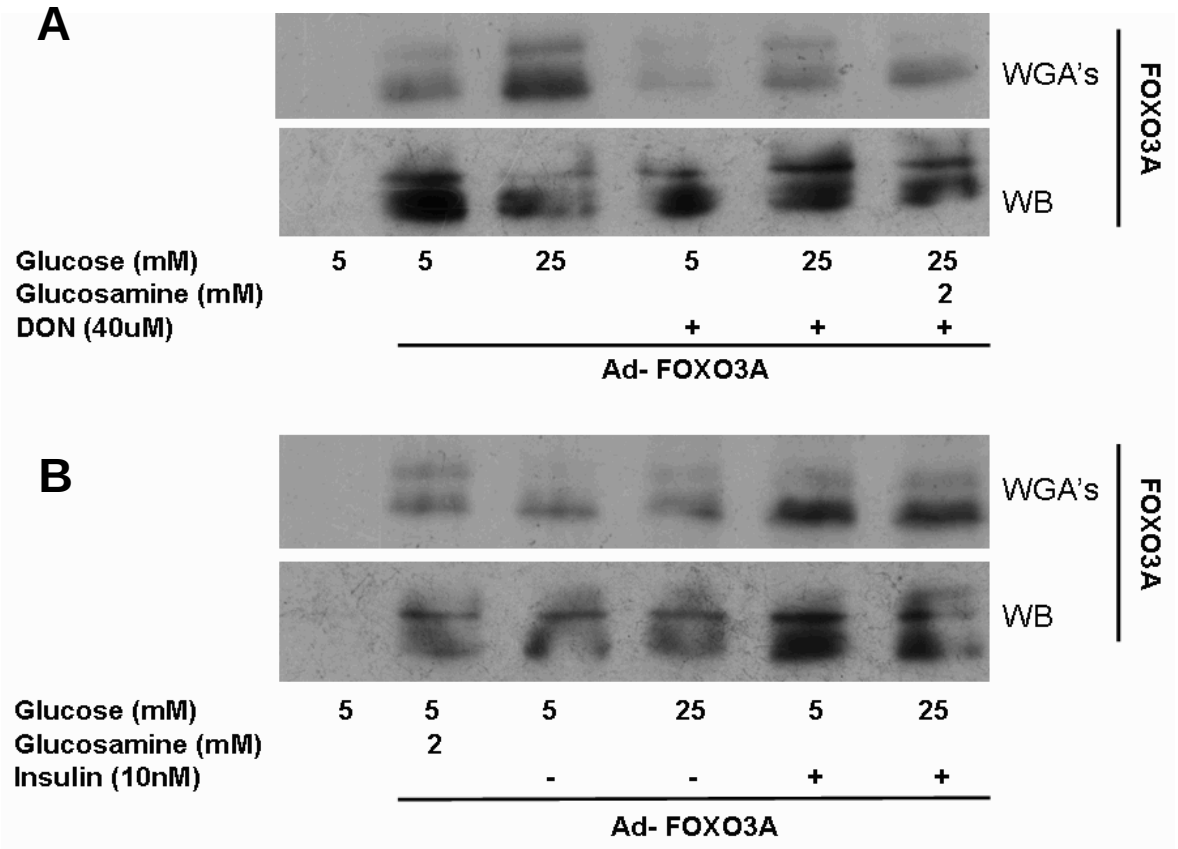


Figure 5.10 Effects of DON on O-GlcNAc modification of FOXO3A

Hepatocytes were untreated or treated with adenoviral vectors for FOXO3A in serum free MEM were added to cells at 2 to 4h after plating and cultured for 4h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 40 μM DON, without or with 10 nM insulin and indicated for determination of protein by immunoblotting assay for FOXO3A .

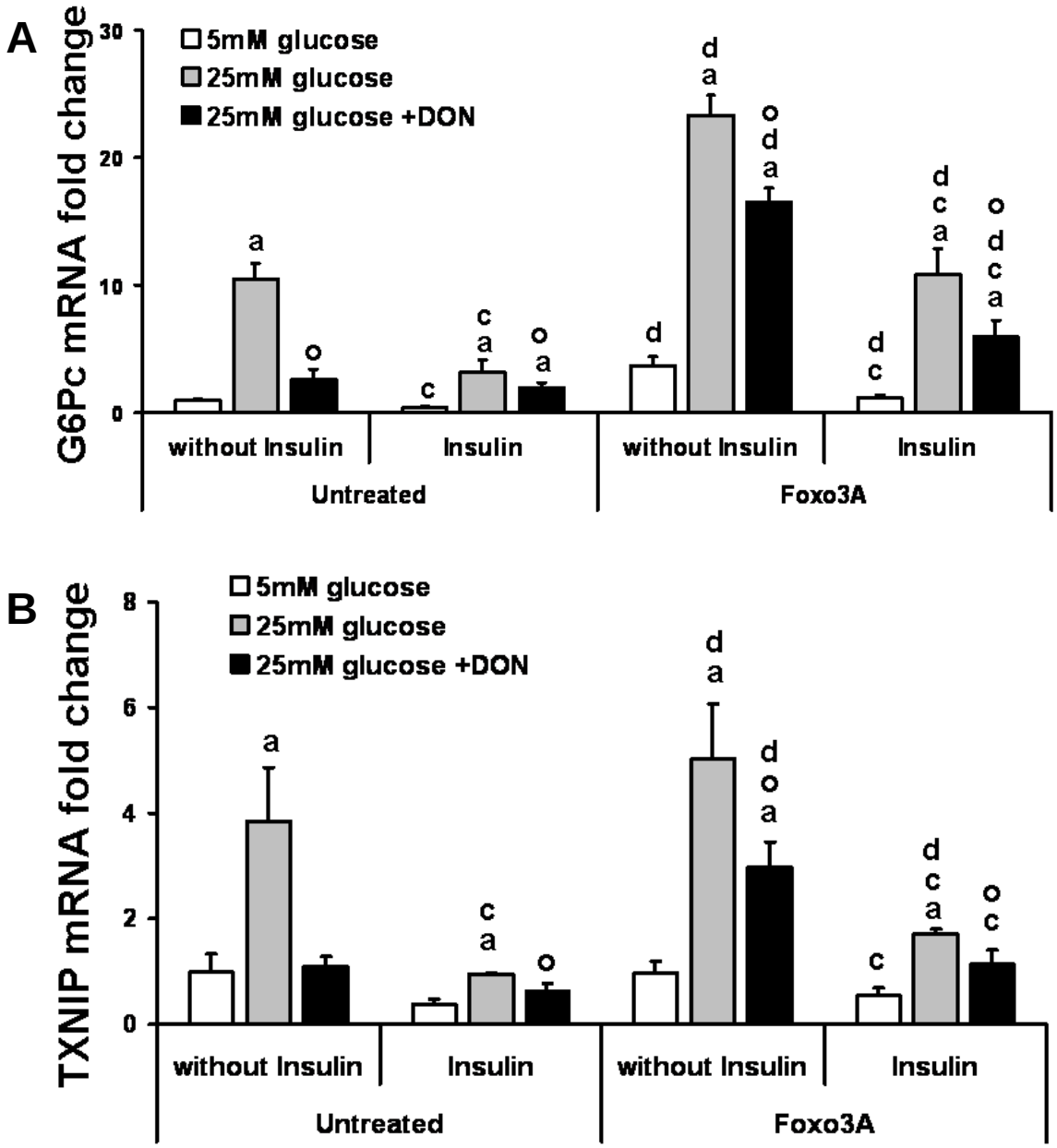


Figure 5.11 Effects of FOXO3A without or with DON on G6Pc and TXNIP mRNA expression

Hepatocytes were untreated or treated with adenoviral vectors for FOXO3A in serum free MEM were added to cells at 2 to 4 h after plating and cultured for 4 h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose and +/- DON. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and 40 µM DON, without or with 10 nM insulin and indicated for determination of gene expression, (A) G6Pc, (B) TXNIP. Mean ± SEM, 3 experiments, duplicate treatments (n=6) $^{a}P< 0.05$ effect of glucose, $^{c}P< 0.05$ effect of insulin and $^{d}P< 0.05$ effect of FOXO3A $^{o}P< 0.05$ effect of DON.

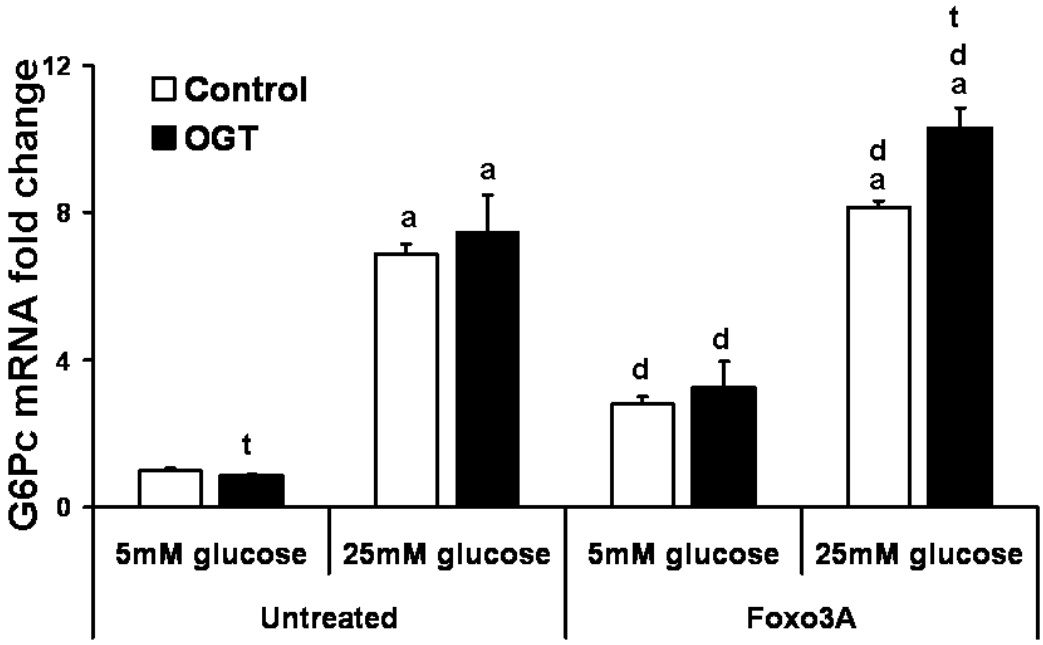


Figure 5.12 Effects of FOXO3A and OGT on G6Pc mRNA expression

Hepatocytes were untreated or treated with adenoviral vectors for FOXO3A and OGT in serum free MEM were added to cells at 2 to 4 h after plating and cultured for 4 h after cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose and indicated for determination of G6Pc mRNA levels, 1 experiments, triplicate treatments (n=3). Mean ± SEM, $^{a}P< 0.05$ effect of glucose, $^{d}P< 0.05$ effect of FOXO3A $^{t}P< 0.05$ effect of OGT.

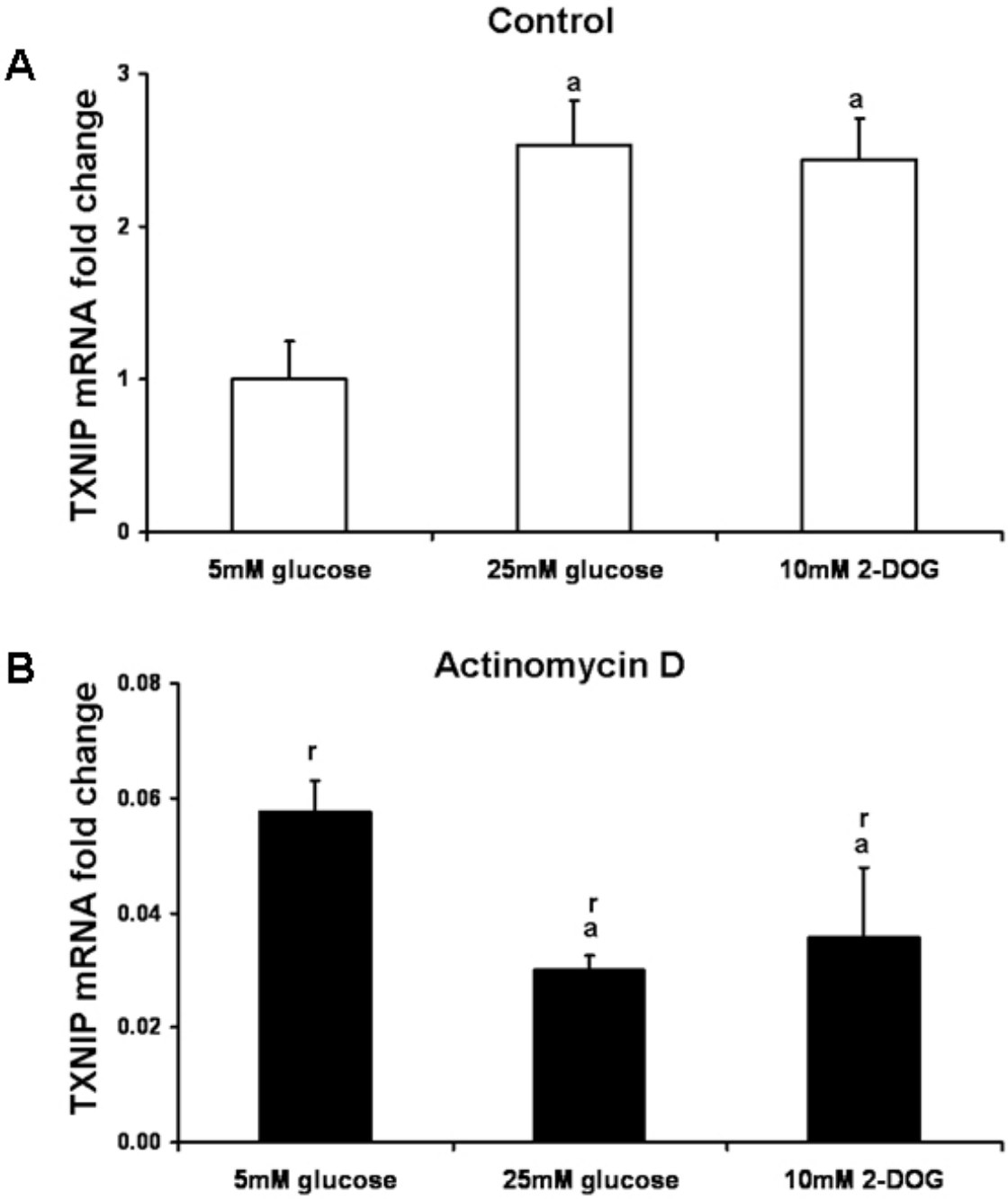


Figure 5.13 Actinomycin D lowers basal TXNIP mRNA and the stimulation by glucose and 2-DOG

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5mM and 25 mM glucose and10 mM 2-deoxyglucose (2-DOG), without or with 5 µg/ml actinomycin D and indicated for determination of TXNIP mRNA levels, 1 experiments, triplicate treatments (n=3). Mean ± SEM, $^{a}P< 0.05$ effect of glucose, $^{r}P< 0.05$ effect of actinomycin D.

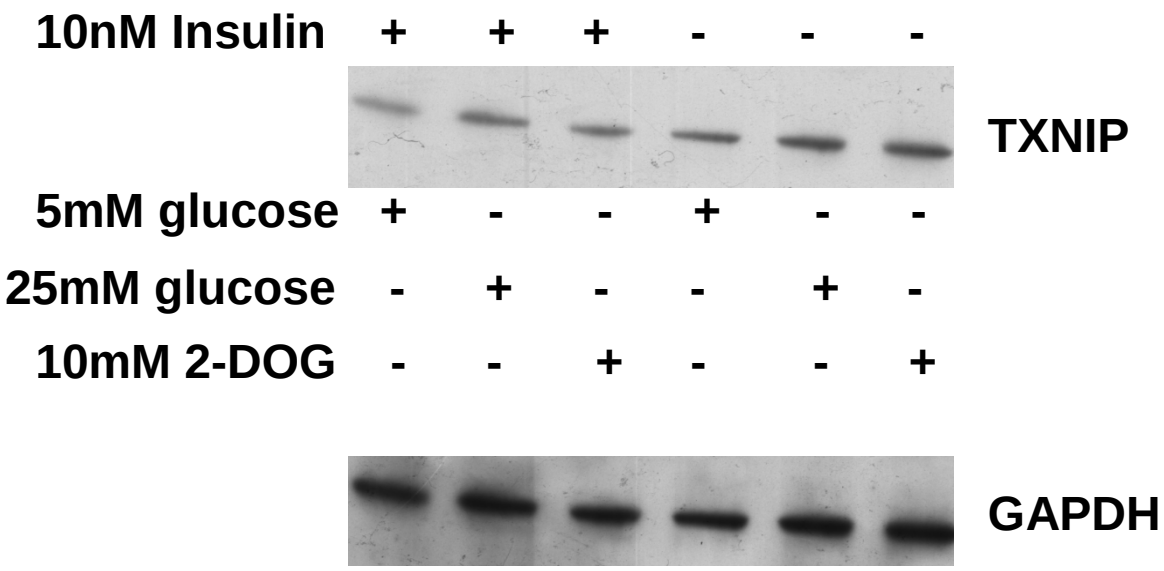


Figure 5.14 Effects of glucose and 2-DOG withou or with insulin on TXNIP protein expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM glucose, 25 mM glucose and 10 mM 2-deoxy-glucose (2-DOG), with or without insulin and determination of protein by immunoblotting assay for TXNIP and GAPDH

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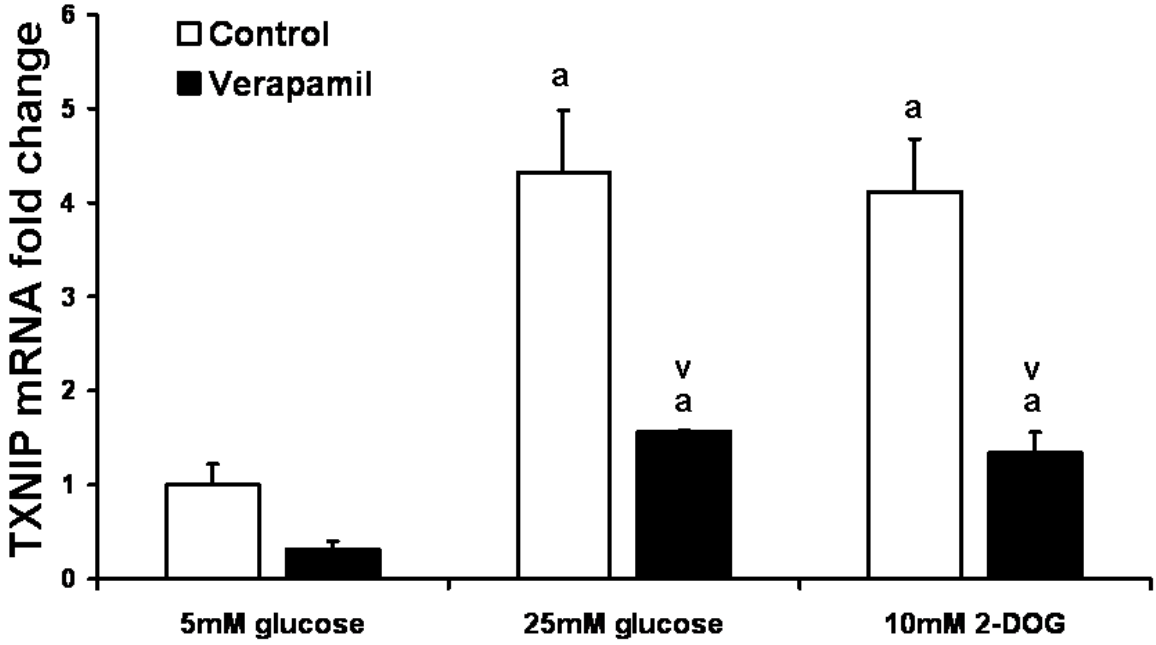

Figure 5.15 Effects of verapamil on TXNIP mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5mM and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG), without or with 150 μM verapamil and indicated for determination of TXNIP mRNA levels, 1 experiments, triplicate treatments (n=3). Mean ± SEM, $^{a}P< 0.05$ effect of glucose, $^{v}P< 0.05$ effect of verapamil.

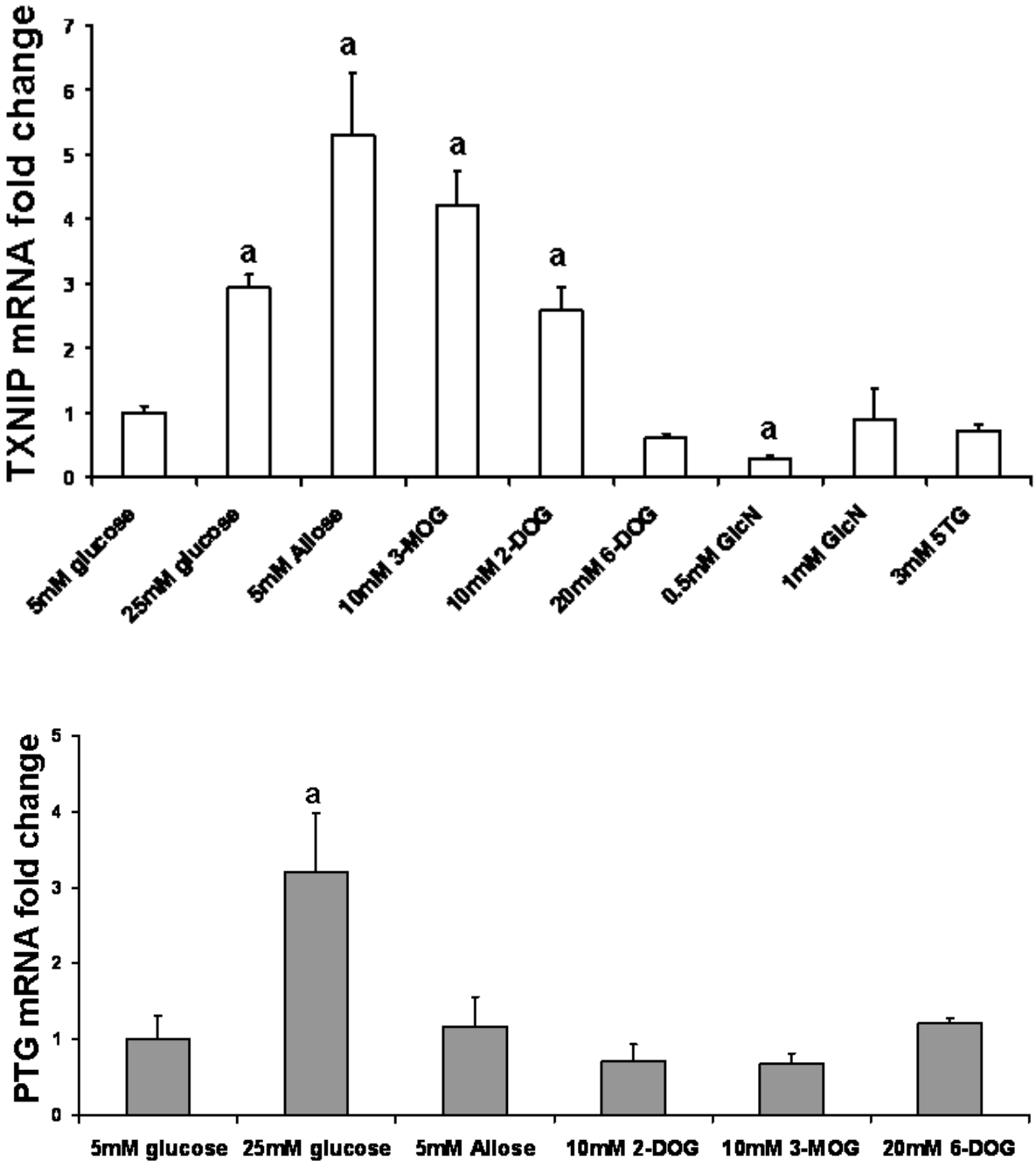

Figure 5.16 Effects of sugars on TXNIP and PTG mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose, 5 mM allose, 10 mM 2-deoxyglucose (2-DOG), 10 mM 3-O-Methyl-glucose (3-MOG), 20 mM 6-deoxyglucose (6-DOG), 0.5 and 1 mM glucosamine (GlcN) and 3 mM 5-thioglucose (5TG) and indicated for determination of TXNIP mRNA levels, 2-8 experiments, duplicate treatments (n=4-16). Mean ± SEM, $^{a}P< 0.05$ effect of glucose.

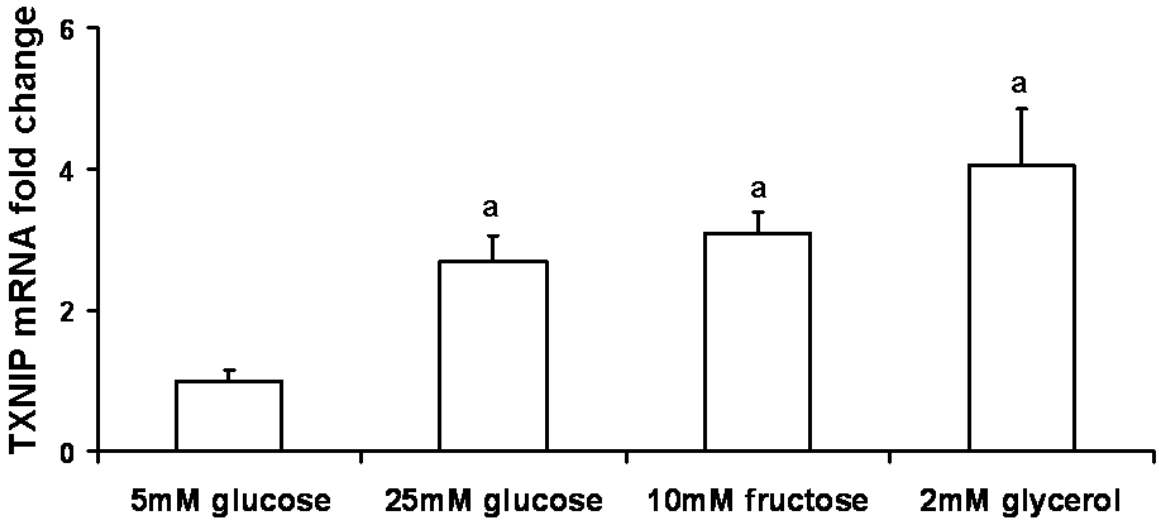

Figure 5.17 Effects of fructose and glycerol on TXNIP mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose, 10 mM fructose and 2 mM glycerol and indicated for determination of TXNIP mRNA levels, 4 experiments, duplicate treatments (n=8) . Mean ± SEM, $^{a}P< 0.05$ effect of glucose.

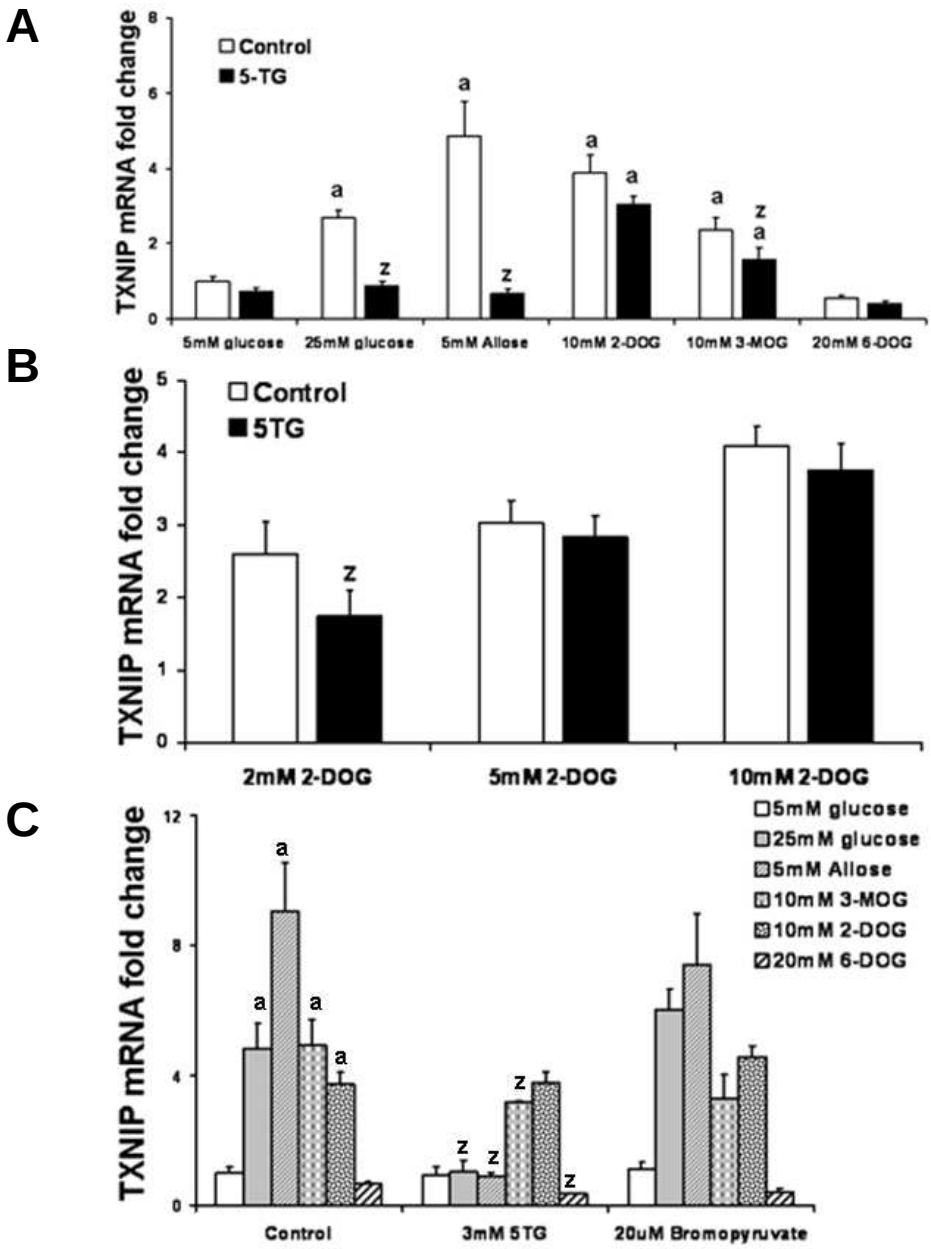

Figure 5.18 Effects of 5-thioglucose (5TG) on TXNIP mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose, 5 mM allose, 10 mM 2-deoxyglucose (2-DOG), 10 mM 3-O-Methyl-glucose (3-MOG), 20 mM 6-deoxyglucose (6-DOG), without or with 3 mM 5-thioglucose (5TG) (A), 2, 5 and 10 mM 2-deoxyglucose (2-DOG) without or with 3 mM 5-thioglucose (B), 5 mM and 25 mM glucose, 5 mM allose, 10 mM 2-deoxyglucose (2-DOG), 10 mM 3-O-Methyl-glucose (3-MOG), 20 mM 6-deoxyglucose (6-DOG), without or with 3 mM 5-thioglucose (5TG) and 20 μM bromopyruvate (C) and indicated for determination of TXNIP mRNA levels, 2-8 experiments, duplicate treatments (n=4-16). Mean ± SEM, $^{a}P< 0.05$ effect of glucose and $^{z}P< 0.05$ effect of 5TG.

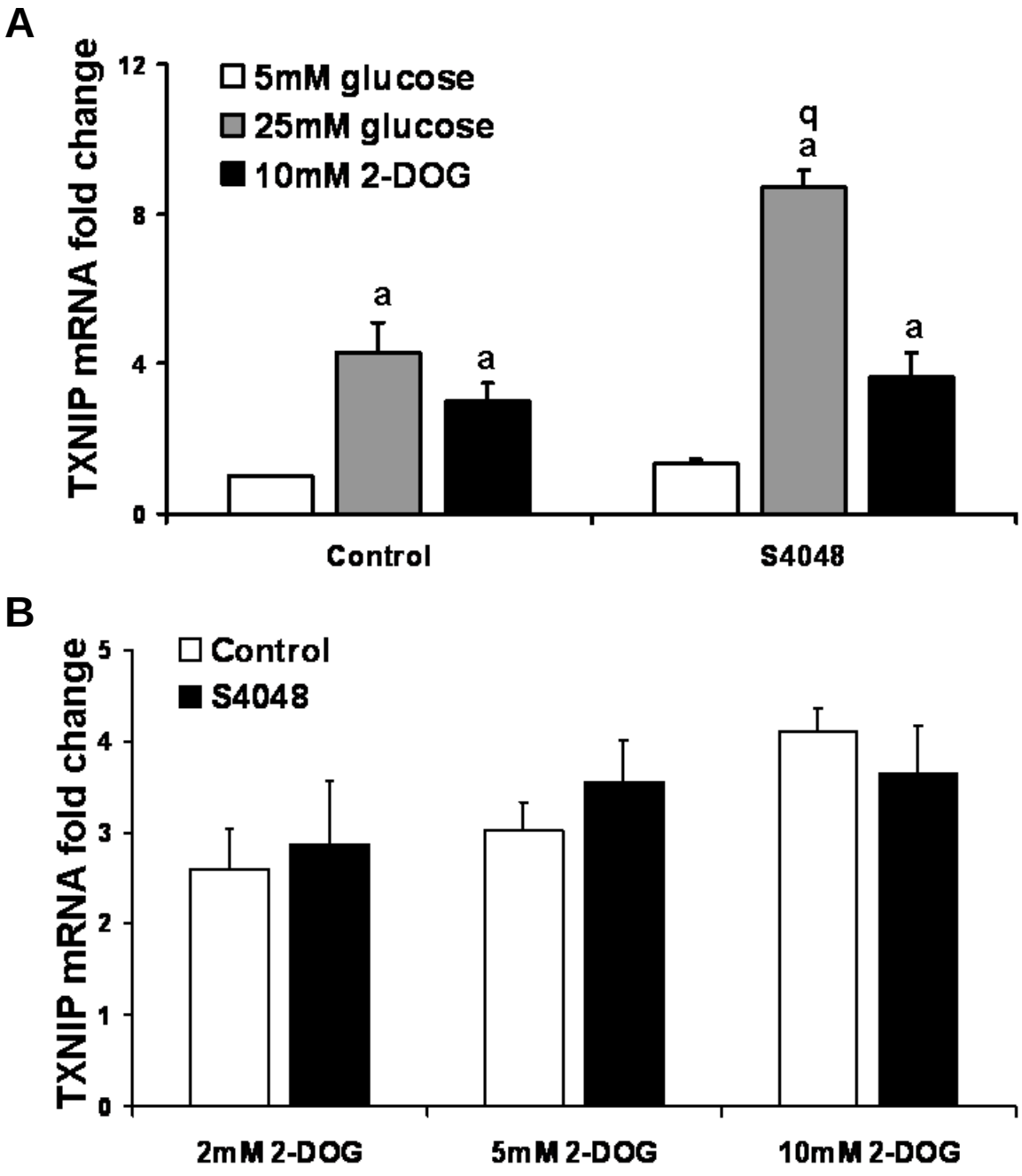

Figure 5.19 Effects of S4048 on TXNIP mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose and 2, 5 and 10 mM 2-deoxyglucose (2-DOG) without or with S4048 and indicated for determination of TXNIP mRNA levels, 6 experiments, duplicate treatments (n=12). Mean ± SEM. $^{a}P< 0.05$ of glucose, $^{q}P< 0.05$ effect of S4048

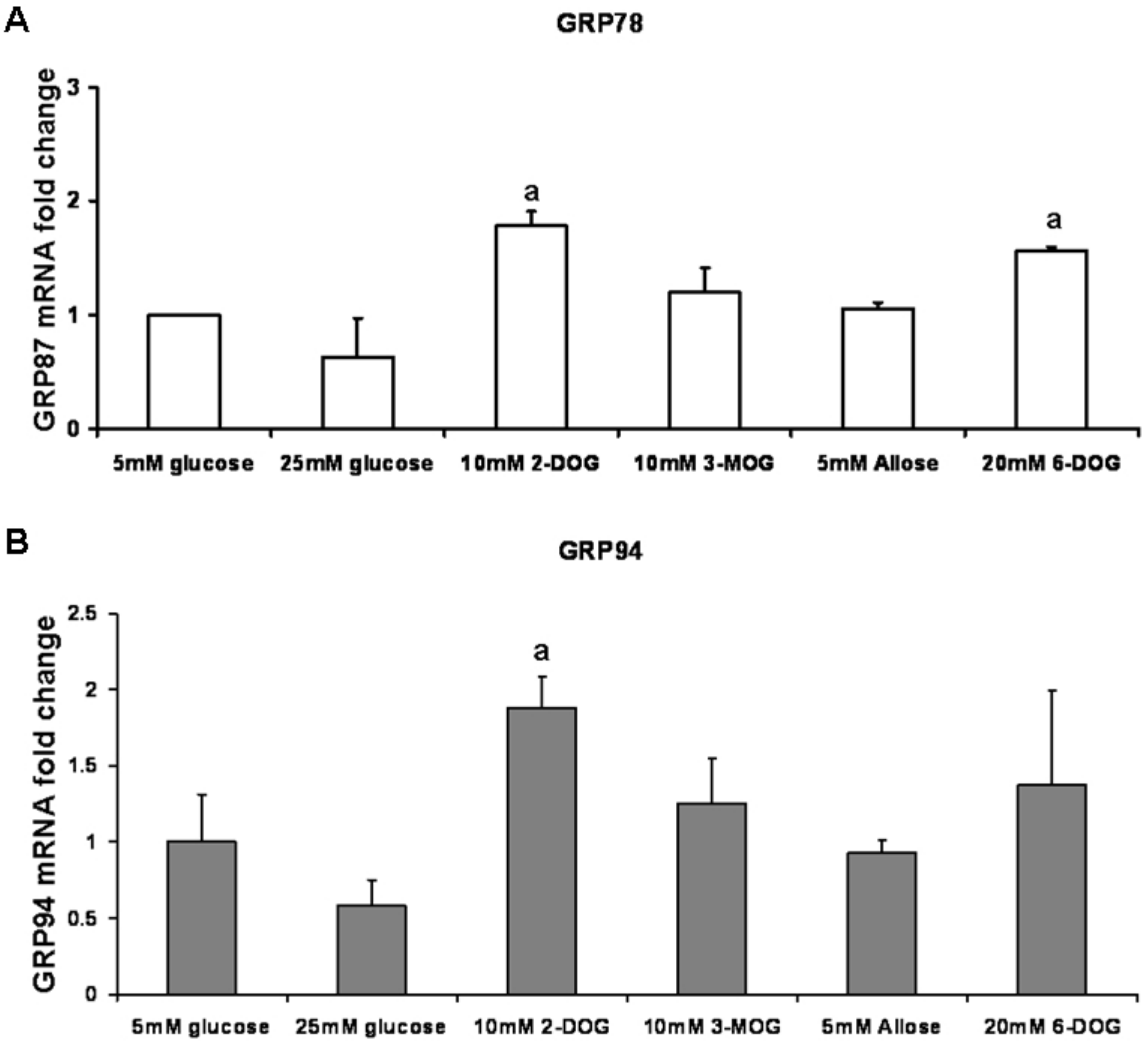

Figure 5.20 Effects of sugars on GRP78 and GRP94 mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 mM and 25 mM glucose, 5 mM allose, 10 mM 2-deoxyglucose (2-DOG), 10 mM 3-O-Methyl-glucose (3-MOG) and 20 mM 6-deoxyglucose (6-DOG) and indicated for determination of GRP78 (A) and GRP94 (B) mRNA levels, 2-8 experiments, duplicate treatments (n=4-16). Mean ± SEM, $^{a}P< 0.05$ effect of glucose.

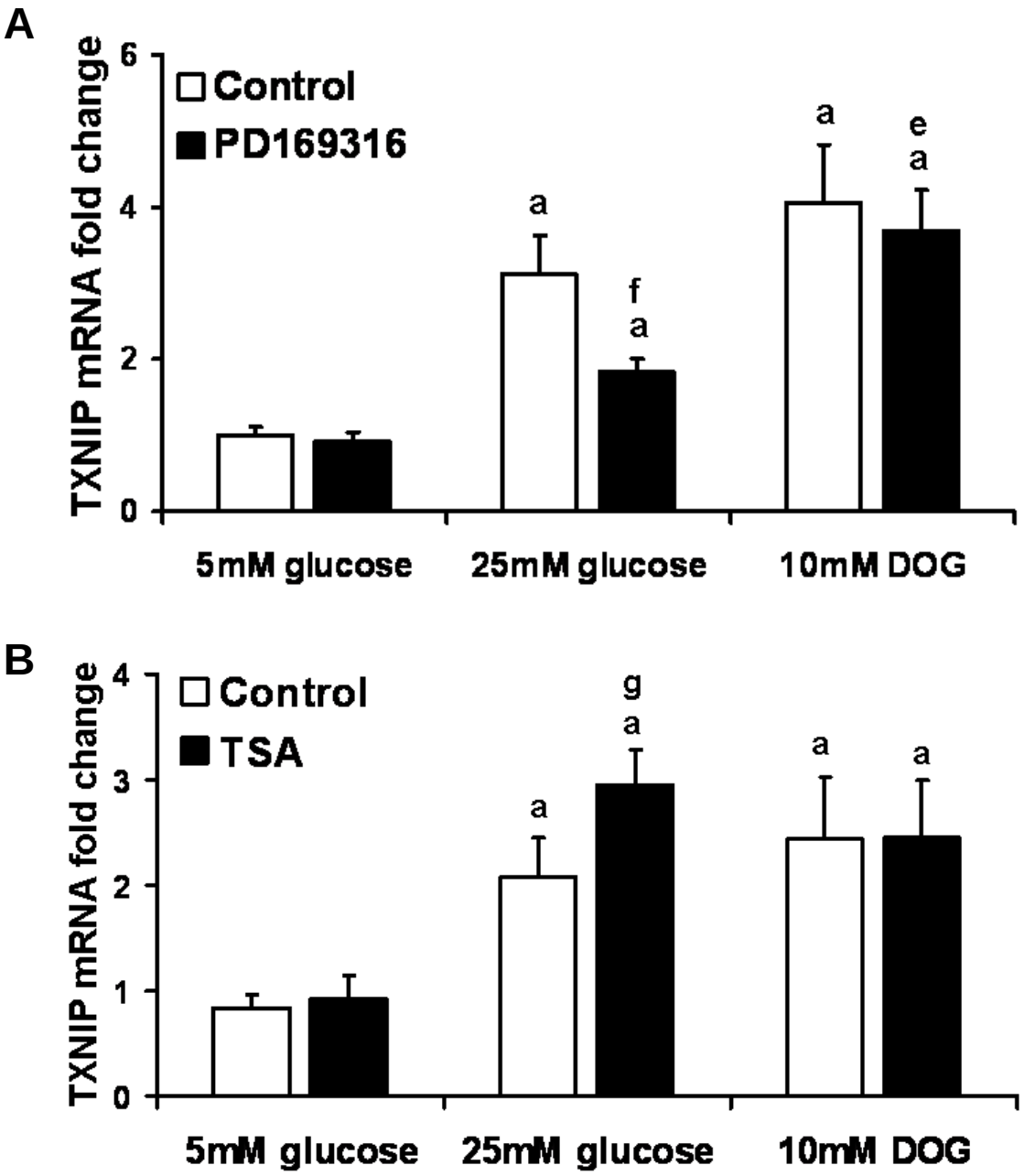

Figure 5.21 Effects of PD and TSA on TXNIP mRNA expression

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing 5 and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG) without or with 100 nM PD169316 (A) and 60 nM TSA (B) and indicated for determination of TXNIP mRNA levels, 3 experiments, duplicate treatments (n=6). Mean ± SEM $^{a}P<$ 0.05 effect of glucose, $^{e}P<$ 0.05 effect of DOG $^{f}P<$ 0.05 effect of PD, $^{g}P<$ 0.05 effect of TSA

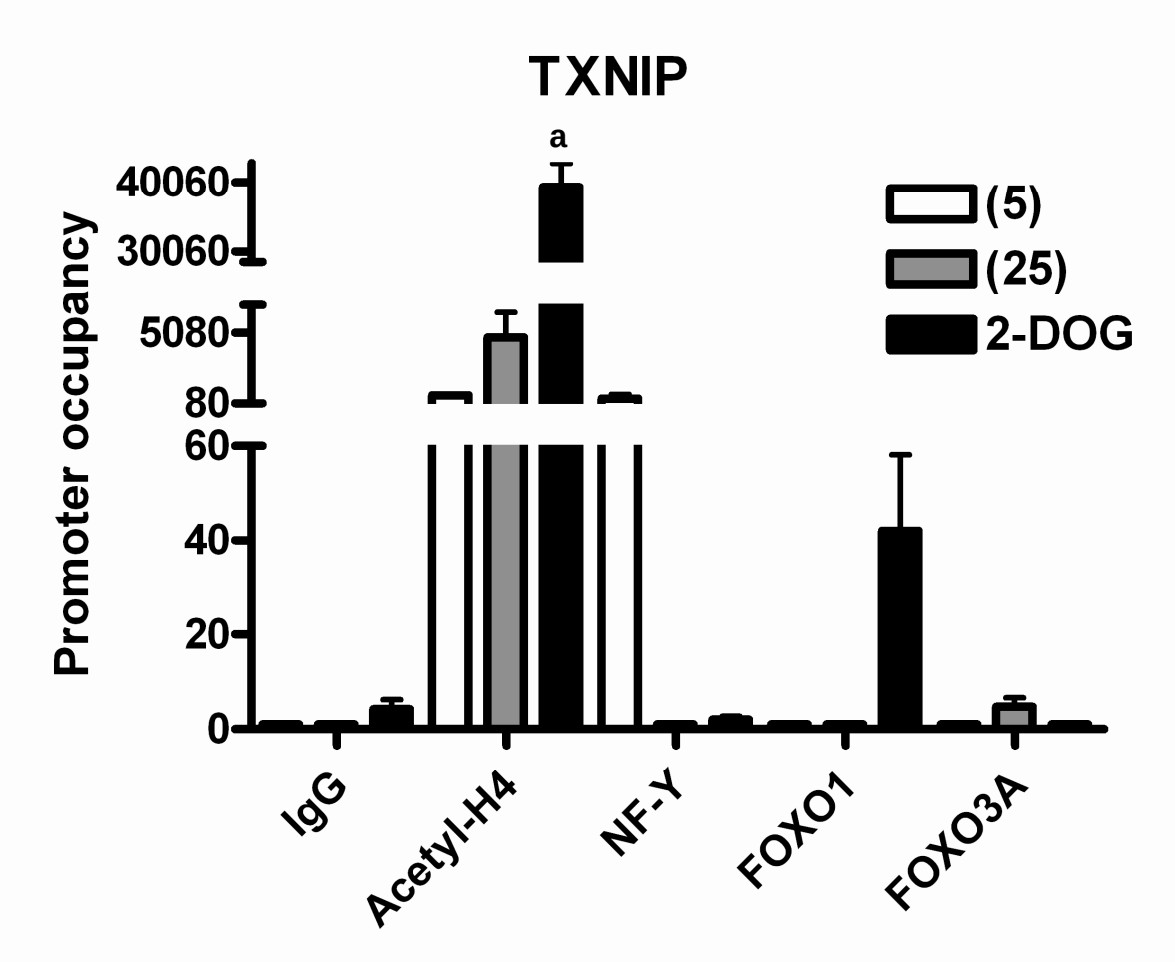

Figure 5.22 Effect of glucose and 2-deoxyglucose (2-DOG) on recruitment of Acetyl-H4, NF-Y, FOXO1 and FOXO3A to the TXNIP promoter

Hepatocyte monolayers were pre-cultured overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 4 h in MEM containing (5) and (25) mM glucose and 10mM 2-deoxyglucose (2-DOG) and 30mins in final incubation with 10nM insulin. ChIP assays were performed as described in the Methods section using control IgG or antibody to Acetyl-H4, NF-Y, FOXO1 or FOXO3A for determination of binding of these proteins to established ChRE elements in the TXNIP promoters.Results are expressed relative to control IgG, 5mM glucose. Mean ± SEM, 3 experiments, $^{a}P< 0.05$ effect of glucose

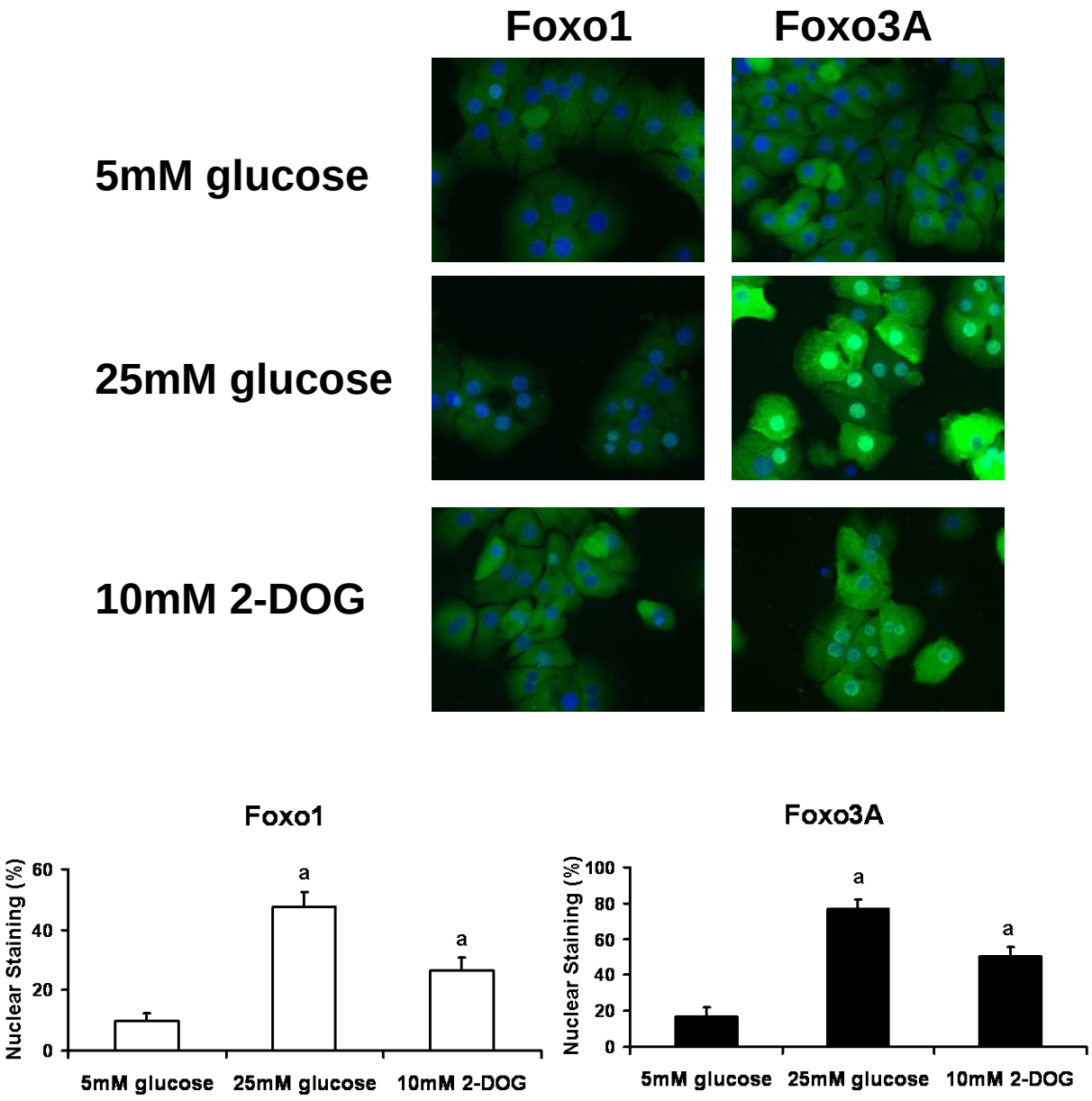

Figure 5.23 Effects of 2-DOG on translocation of FOXO1 and FOXO3A

Hepatocyte were plating and cultured for overnight in MEM containing 10 nM dexamethasone, 5 mM glucose. They were then incubated for 30 min in MEM containing 5 mM and 25 mM glucose and 10 mM 2-deoxyglucose (2-DOG), with 1h incubation of 10 nM insulin for FOXO1 and 15 min incubation of 10 nM insulin for FOXO3A. Cells were scored as having greater levels of FOXO1 and FOXO3A in the cytoplasm or nucleus as described in methods. Images representative of 5 experiments, coverslips treated in duplicate (n=10). $^{a}P< 0.05$ effect of glucose.

Chapter 6

General Conclusions

General Conclusions

Chronic hyperglycaemia in type 2 diabetes causes various secondary complications including insulin resistance by mechanisms that are often described as glucotoxicity. The HBP and modification of proteins by O-GlcNAc have both been suggested to be involved in development of insulin resistance by glucose toxicity in diabetes (Whelan et al., 2008; Yang et al., 2008; Vosseller et al., 2002; Arias, 2004). Since 1980, more research studies have been published on the role of hexosamine pathway through modification of proteins by O-GlcNAc in diabetes and to understand the mechanism by which O-GlcNAc modification of proteins affects their function (Copeland et al., 2008; Teo et al., 2010). In addition, O-GlcNAc plays an important role in other chronic diseases such as cardiovascular and cancer (Hart et al., 2011). Marshall first reported the correlation between the HBP and insulin resistance in adipocytes (Marshall et al., 1991), and proposed that increased flux through the HBP causes hyperlipidaemia and insulin resistance. This raised important questions on the role of the HBP in the liver (Veerababu et al., 2000). The HBP leading to formation of UDP-GlcNAc and covalent modification of proteins by addition of O-GlcNAc is thought to be a mechanism for switching off insulin signalling by prolonged hyperglycaemia (Hart et al., 2011). Various mechanisms by which O-GlcNAc modification of proteins can regulate insulin signalling have been reported including: protein tyrosine phosphatases (PTPases) which reverse the effect of tyrosine kinases; phosphoinositide phosphatase which reverse the effect of PI-3-kinase (Akt/PKB) and serine/threonine phosphorylation of IRS proteins which counteracts the tyrosine phosphorylation of these substrates (Yang et al., 2008). Akt/PKB

activated phosphorlyation of FOXO which causes exclusion from the nucleus and decreased transcription of gluconeogenic genes.

In the liver several genes are regulated at the transcriptional level by both insulin and glucose. Enzymes of glycolysis (e.g. pyruvate kinase) and lipogenesis (ACC and FAS) are induced by both insulin and glucose, by activation of SREBP-1c and ChREBP-Mlx, respectively. The increased expression of lipogenic enzymes in Type 2 diabetes is thought to be due to increased activation of both SREBP-1c and ChREBP-Mlx because knock down of both SREBP-1c and ChREBP improves the lipogenic defect. Type 2 diabetes is also associated with increased hepatic glucose production and increased expression of G6Pc, which catalyses the final step in hepatic glucose production. This enzyme is repressed by insulin and induced by glucagon and it is induced by glucose. FOXO transcription factors are major regulators of the repression of G6Pc by insulin (Onuma et al., 2006) and various studies have proposed that the glucose induction of G6Pc is mediated by covalent modification by O-GlcNAc of either FOXO transcription factors (Kuo et al., 2008; Housley et al., 2009) which are regulated by insulin or transcription factors that are regulated by glucagon such as CRTC2, the binding protein of CREB (Dentin et al., 2008); or co-regulators of transcription such as PGC-1α (Housley et al., 2009). Evidence for covalent modification of transcription factors involved in G6Pc regulation by O-GlcNAc has been obtained from studies with GlcN (generally at concentrations of 5 to 10 mM) and from use of inhibitors of GFAT (DON) and OGA (PUGNAc) (Dentin et al., 2008; Kuo et al., 2008; Housley et al., 2009). UDP-NAG the substrate for covalent modification of proteins by OGT is generated by the HBP.

Recent work in our laboratory had shown that the transcription factor ChREBP-Mlx which is widely implicated in the regulation of enzymes of glycolysis and

lipogenesis is also a target gene for ChREBP (Arden et al., 2012). This suggests that there are various mechanisms by which glucose can induce G6Pc: (i) through ChREBP activation by a similar mechanism as enzymes of glycolysis and lipogenesis; (ii) through covalent modification by O-GlcNAc of other transcription factors that are regulated by insulin and glucagon; (iii) through inhibition of insulin signalling by O-GlcNAc. The aim of this study was to determine the role of the HBP and O-GlcNAc modification mechanisms in glucose regulated gene expression of G6Pc. The focus of this study was G6Pc and not other ChREBP targets because of the major role of this enzyme in hepatic glucose metabolism and also because G6Pc unlike most other ChREBP targets is regulated oppositely by insulin and glucose. Another gene that is regulated oppositely by glucose and insulin in extrahepatic tissues is TXNIP. Studies on subjects with type 2 diabetes identified elevated expression of this gene in muscle of diabetics and showed that increased TXNIP expression inhibits glucose uptake by adipocytes (Mootha et al., 2007). TXNIP was therefore also studied in parallel with G6Pc to test whether the HBP is involved in glucose regulation of this gene.

6.1 The study of the HBP in hepatocytes

In order to study the relative roles of ChREBP-Mlx and O-GlcNAc modification in regulation of gene expression it was first necessary to establish valid tools that are specific for the HBP and / or O-GlcNAc modification that do not interfere with other mechanisms of glucose signalling. The most commonly used method for increasing HBP flux and covalent modification with O-GlcNAc is incubation with 5-10 mM GlcN or incubation with the OGA inhibitor, PUGNAc and overexpression of OGT to increase covalent modification of proteins without

changing HBP flux. Inhibitors of GFAT (AZN and DON) or of OGT (BADGP) are also used to inhibit HBP flux and OGT activity.

This study tested the effects of various GlcN concentrations and confirmed that GlcN is rapidly metabolised by the HBP from the measurement of NAG metabolites which accumulated within 4 h incubation to much higher levels than occur with 25 mM glucose. Unlike 25 mM glucose which increased the concentration of NAG-metabolites but not of GlcN 6-P, concentrations of GlcN at 5mM or above caused a large accumulation of GlcN 6-P showing that at these concentrations of GlcN the activity of GlcN 6-P NATase becomes rate limiting and its substrate accumulates. This was associated with inhibition of glucose phosphorylation, and accumulation of glucose 6-P and also activation of phosphorylase independently of the lowering of glucose 6-P. Titration with a range of concentrations of GlcN showed that the minimum concentration that raises NAG metabolites (0.5 mM) also caused inhibition of glucose phosphorylation and lowering of both glucose 6-P and $F2,6P_2$. Therefore GlcN could not be used as a tool to increase HBP flux without interfering with other metabolic pathways.

Experiments with GFAT inhibitors established that DON could be used at a concentration of 40 μM to inhibit GFAT. At this concentration, the inhibitor had negligible effects on glucose phosphorylation and protein synthesis and it did not affect $F2,6P_2$ which is a regulator of ChREBP-Mlx and MondoA-Mlx. Therefore DON was established to be a valid tool to inhibit flux through the HBP with negligible effects on other metabolic pathways. Experiments on protein labelling with ^{14}C-glucose and without or with DON to inhibit HBP allowed the estimation of flux through the HBP resulting in the labelling of proteins. This gave an estimate for HBP flux of 3% of the rate of glucose phosphorylation. The

effectiveness of PUGNAc and BADGP as inhibitors of OGA and OGT was tested from protein labelling with GlcN. These experiments did not show increased labelled with PUGNAc but showed inhibition with BADGP. However this inhibitor unlike the GFAT inhibitor inhibited protein synthesis and was therefore considered to be non-specific. These studies therefore only provided support for use of the GFAT inhibitor.

6.2 Control of G6Pc and TXNIP gene expression by glucose

This study showed that expression of TXNIP in hepatocytes like expression of G6Pc was inhibited by insulin and induced by glucose. In the presence of insulin the induction by glucose of both genes was much smaller than in presence of insulin. This indicates that insulin inhibits the expression of these genes both independently of the high glucose stimulation and also possibly by inhibiting the glucose stimulation. Experiments with a dominant-negative variant of Mlx (the partner of both ChREBP and MondoA) showed that the glucose induction of both G6Pc and TXNIP in the absence of insulin was largely Mlx-dependent.

The Mlx-dependence of the glucose induction of G6Pc and TXNIP is consistent with the findings from the study by Ma (Ma et al., 2006) which was the first study to determine the role of Mlx in glucose regulation of gene expression. Experiments with ChREBP or FOXO3A overexpression confirmed that both these transcription factors cause induction of G6Pc and TXNIP and this induction of both genes was greater in the presence of glucose suggesting that both transcription factors mediate the effect of glucose.

An interesting finding from this study was the converse effects of insulin and glucose on the subcellular location of both FOXO1 and FOXO3A. The available

antibody to FOXO1 detected the endogenous protein. However, overexpression of FOXO3A was necessary to measure immunoactivity and translocation. The available results are therefore for endogenous FOXO1 and overexpressed FOXO3A. Insulin caused rapid translocation of FOXO1 from the nucleus to the cytoplasm (> 60% within 30 min) and almost total exclusion of FOXO3A from the nucleus within 15 min. Whether the difference between FOXO1 and FOXO3A is a true isoform difference or the result of overexpression of FOXO3A could not be determined. However, interestingly, high glucose had clear and opposite effects from insulin on the subcellular location of both FOXO1 and FOXO3A. High glucose inhibited the effect of insulin on FOXO1 translocation and caused the retention of FOXO1 in the nucleus and it caused increased accumulation of FOXO3A in the nucleus in the absence of insulin. Athough the studies on FOXO1 could not distinguish between effects of glucose on the insulin mechanism and effects of glucose that are independent of insulin, because in these conditions the protein was present exclusively in the nucleus, the studies on FOXO3A suggest that glucose causes translocation of the protein into the nucleus independently of insulin action.

6.3 Role of the HBP in glucose-regulation of G6Pc and TXNIP gene expression

The starting point for this project were the reports from various research groups that modification of transcription factors by O-GlcNAc is the major mechanism by which glucose induces G6Pc (Dentin et al., 2008; Housley et al., 2008; Housley et al., 2009; Kuo et al., 2008). In particular the study by Dentin on primary hepatocytes showed that GlcN causes a larger induction of G6Pc than 25

mM glucose. The present study could not replicate an effect of GlcN on the induction of G6Pc. For these experiments GlcN was tested over a range of concentrations and in different hormone conditions. Interestingly an earlier paper by Masillon (2002) that used Northern blots for visualisation of G6Pc mRNA showed stimulation by both xylitol and glucose but inhibition of the glucose stimulation by 2 mM GlcN, in agreement with the present findings. In this study GlcN (0.5 mM) inhibited the stimulation by 25 mM glucose. This effect is most likely explained by the inhibition of glucose phosphorylation by GlcN and the lowering of $F2,6P_2$ which has been shown to be essential for glucose induced activation of ChREBP (Arden et al., 2012; Petrie et al., 2013).

The experiments with the GFAT inhibitor DON, provided clear evidence for inhibition of the glucose-induction of both G6Pc and TXNIP. This effect of DON is most likely explained by inhibition of GFAT because it was not observed in the presence of 0.5 mM glucosamine which increase flux through GFAT.

The inhibitory effect of DON on G6Pc gene expression was associated with decreased nuclear staining of ChREBP, which was also associated with lower ChREBP protein as shown by immunoblotting. DON had no effect on the increase in $F2,6P_2$ caused by glucose indicating that it does not interfere with the ChREBP translocation signalling metabolite However whether in addition to a decrease in ChREBP protein there is also a defect in the translocation could not be determined from the present experiments because the immunostaining studies are based on a count of stained nuclei and not on the relative distribution of staining in the nuclei and cytoplasm, because the cytoplasmic fluorescence of hepatocytes is in part due to non-specific autofluorescence.

The studies testing the role of the HBP on the glucose stimulation of translocation of FOXO transcription factors showed a strong inhibitory effect of the GFAT inhibitor on translocation of FOXO3A without a lowering of FOXO3A protein immunoactivity. They also showed inhibition with GFAT inhibitor of covalent modification of FOXO3A by O-GlcNAc in the WGA-precipitation assays. Further evidence supporting a role for increased activation of G6Pc by FOXO3A by O-GlcNAc modification was obtained by combined expression of FOXO3A and OGT which caused a greater increase in G6Pc expression. Together the results from this thesis support a role for increased modification of FOXO3A by O-GlcNAc in contributing to the raped glucose induction of G6Pc expression.

6.4 Regulation of TXNIP by glucose analogues

An interesting finding from this study was that glucose analogues which have been shown previously to induce TXNIP in other cell types (Pedersen and Ayer, 2011) were also found to be inducers of TXNIP but not of G6Pc or PTG, showing clearly that regulation by TXNIP differs from that of both ChREBP targets (G6Pc) and also MondoA targets (PTG). Interestingly, the induction of TXNIP by 2-DOG unlike the induction by glucose was not Mlx-dependent, further confirming that this mechanism does not involve with ChREBP-Mlx or MondoA-Mlx. However, experiments with actinomycin D confirmed that the induction by 2-DOG was due to a transcriptional mechanism. 3-MOG and allose but like 2-DOG also induced TXNIP, and the effect of theses analogues was inhibited by the hexokinase inhibitor 5TG suggesting that it is most likely mediated by the phosphorylated form of these sugars as proposed in the recent study by Pedersen (Pedersen and Ayer, 2011). In this study 6-DOG and GlcN had no effect on

induction of TXNIP, because 6-DOG cannot be phosphorylate this further supports that the mechanism of TXNIP activation is most likely mediated by a phosphorylated intermediate. However, it is clearly mediated by a different mechanism from glucose because it is Mlx-independent.

Other different binding the TXNIP induction by glucose compared with 2-DOG pointed to a role of histone acetylation. The induction of TXNIP by 2-DOG was not further inhibited by the HDAC inhibitor and it was associated with increased binding of Acetyl-H4 to the TXNIP promoter by 2-DOG indicated a role for acetylation with activation of TXNIP by glucose analogues.

6.4 Summary

1. In summary this study shows that the induction of G6Pc by glucose is inhibited by both lowering of F2,6P_2 with a kinase-deficient variant of 6-phosphfructo-2-kinase/fructose-2,6-bisphosphatase 1 (PFK-KD) which had no effect on NAG-metabolites and by inhibition of the HBP with the GFAT inhibitor which had no effect on F2,6P_2. This indicates that at least two independent metabolite signalling pathways are involved in the glucose induction of G6Pc: elevation in F2,6P_2 which causes translocation of ChREBP and flux through the HBP which causes covalent modification of FOXO transcription factors and accumulation in the nucleus.

2. Regulation of TXNIP by glucose that regulation of G6Pc was also dependent on flux through the HBP. However, glucose analogues also induced TXNIP by a mechanism involving histone acetylation.

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Appendix I: TXNIP proximal promoter sequence and ChIP primers

AGAAACAATAGCTGTGCAGGGAACAAGACACAGAACTGTCCACGGGGCTTTTCTTAATTA
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CCCAAGTCAACACCCTCTGACCCTTAGCGGCCTCCTCTCTTCCTTTCAGATCTCAAGATTTA
AAAACAATAGTAACTTTTTACATCTAAAGAGGTTATTTCGGGTGTCTCTGGAGTGAGTACT
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GAACGAGTTTCAGTCCGAGAACCAAAATGGCAGCTTACAACTCTTGACTCCAGTTCTAAG
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AATGAATTGCGCTGGCTAAGACAGGCATGAAATCCTCTCCTAAGCACATTTTTCTTTTACC
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CTGCCCCAGGCCCCGCCCCTCCTCCCTGGCAAGGCTG**CGCACCCGAACAACAACCAT**TTT
CCCCGCTAAGAG**CACACTGTGTCCACGCG**CCTCTGCGGCCTCGCTGATT**GGTTA**GAGGC
CTG**GTAAACAAG**GACCAAGTAG**CCAAT**GGGAGAACTGTG**CACGAGGGATGCACGAG**CC
TCCGGGCCAGCACTCGCGTGGAGCGTCAAGCCAGGCGGCTATATAATG**CCGTTTCCGGC**
TCCCGCTTGACACTCTCCTCTTCTGGTCTTTGGATATCCAGAGTTCCTCCAGTTGCGAGAA
AACAGCTGTTATTTTTCTCCTGAAAGCTTTTGGCACAACCAGCTGGTTGAAACTTTCAGGC
ACCTTTTAGAGAAGTTGTTAAGGTTTTGTTTGAGGCTTTCTTTGGGTTTTTAAGCCCTCTCT
GCCTCACGGAGAGACTTAAGTTCTTAGTTTGCTGAGAAGGATTCTGAAGAGTTTTTCCTCT
CCGGCTTCCGTTTTTCTTGAACCCACTCGGCTCAATCATG

ChoRE-a - Ayer
ChoRE-b - Luo
FOXO binding site - Luo
NF-Y binding site - Luo

Luo & Hagen primers