Examining the transcription factor FOXO1 and its regulation by glucocorticoids and inflammatory mediators in hepatic insulin signalling

by

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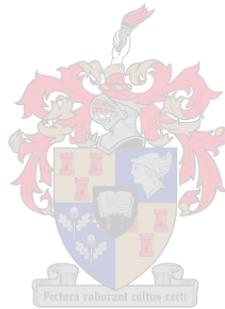
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Declaration

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Abstract

Insulin resistance is the decrease in responsiveness to normal circulating insulin levels, resulting in an attenuated biological response in target tissues (including the liver, muscle, and adipose tissue). In the liver specifically, insulin resistance is no longer able to halt gluconeogenesis, the process whereby glucose production is increased. The forkhead box 1 (FOXO1) transcription factor, the protein responsible for the transcription of enzymes required for gluconeogenesis, remains active in the nucleus. There are many factors that can contribute to the development of insulin resistance, including stress and inflammation. In chronic stress, the hypothalamic-pituitary-adrenal (HPA) axis becomes hyperactive resulting in elevated glucocorticoid (GC) levels such as cortisol (F), whereas chronic inflammation is associated with an increase in the production and secretion of pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6). Both GCs and elevated pro-inflammatory cytokine levels have been implicated in the development of insulin resistance, however, stress and inflammation-induced hepatic insulin resistance is not well understood, particularly the effects stress and inflammation have on the regulation of FOXO1. For this reason, the main aim of this study was to investigate the effects GCs and pro-inflammatory cytokines have on FOXO1 expression and activity in the liver cell line HepG2. The results showed that GCs, as well as pro-inflammatory cytokines impair insulin signalling in liver cells, although to different extents and time of exposure. More specifically, Dexamethasone (Dex) increased FOXO1 mRNA and protein expression and had the most pronounced activity on the promoter of FOXO1, whilst F showed activity on the promoter of FOXO1 but only increased FOXO1 protein expression. Both pro-inflammatory cytokines increased FOXO1 mRNA expression but had no effect on the promoter of FOXO1, and only TNF-α increased FOXO1 protein levels. In addition, TNF-α appeared to act co-operatively with both GCs, increasing FOXO1 mRNA expression, whereas IL-6 inhibited GC-induced effects on FOXO1. Finally, both GCs and pro-inflammatory cytokines prevented insulin from inhibiting FOXO1 activity. To the best of our knowledge, this is the first study to investigate stress- and inflammation-induced insulin resistance on FOXO1 both alone and in combination. The findings of this study suggest FOXO1 dysregulation as being a potential contributor to hepatic insulin resistance induced by stress and inflammation.

Opsomming

Insulienweerstandigheid is die afname in reaksie op normale insulienvlakke in sirkulasie, wat lei tot 'n verswakte biologiese reaksie in teikenweefsels (insluitend die lewer-, spier- en vetweefsel). In die geval van die lewer, kan insulienweerstandigheid nie meer glukoneogenese, die proses waardeur die produksie van glukose verhoog word, stop nie. Die transkripsiefaktor van die vurkkas 1 (FOXO1), die proteïen wat verantwoordelik is vir die transkripsie van ensieme wat benodig word vir glukoneogenese, bly aktief in die kern. Daar is vele faktore wat kan bydra tot die ontwikkeling van insulienweerstandigheid, insluitend spanning en inflammasie. In chroniese stres word die hipotalamus-hipofise-bynier (HPA) hiperaktief, wat lei tot verhoogde glukokortikoïedvlakke (GC vlakke) soos kortisol (F), terwyl chroniese inflammasie geassosieer word met 'n toename in die produksie en afskeiding van pro-inflammatoriese sitokiene soos as tumornekrose faktor α (TNF-α) en interleukien 6 (IL-6). Beide GC's en verhoogde pro-inflammatoriese sitokienvlakke word geïmpliseer in die ontwikkeling van insulienweerstandigheid, maar spanning en inflammasie-geïnduseerde lewerinsulienweerstandigheid word nog nie goed verstaan nie, veral die uitwerking wat spanning en inflammasie op die regulering van FOXO1 het. Om hierdie rede was die hoofdoel van hierdie studie om die effek wat GC's en pro-inflammatoriese sitokiene op FOXO1-uitdrukking en -aktiwiteit in die lewersellyn HepG2 het, te ondersoek. Die resultate toon dat GC's, sowel as pro-inflammatoriese sitokiene, insulienseining in lewerselle benadeel, hoewel in verskillende mate en blootstellingstyd. Meer spesifiek het Dexamethasone (Dex) die FOXO1-mRNA en proteïenuitdrukking verhoog en het die mees beduidende aktiwiteit op die promotor van FOXO1 gehad, terwyl F aktiwiteit getoon het op die promotor van FOXO1, maar slegs die FOXO1-proteïenuitdrukking verhoog het. Beide pro-inflammatoriese sitokiene het FOXO1 mRNA-uitdrukking verhoog, maar het geen effek op die promotor van FOXO1 gehad nie, en slegs TNF-α het FOXO1-proteïenvlakke verhoog. Daarbenewens blyk dit dat TNF-α saamwerk met albei GC's, wat die uitdrukking van FOXO1 mRNA verhoog, terwyl IL-6 GC-geïnduseerde uitwerkinge op FOXO1 inhibeer. Ten slotte het beide GC's en pro-inflammatoriese sitokiene verhinder dat insulien FOXO1-aktiwiteit inhibeer. Na die beste van ons wete, is dit die eerste studie wat insulienweerstandigheid weens spanning en inflammasie op FOXO1, alleen en in kombinasie, ondersoek. Die bevindings van hierdie studie dui daarop dat FOXO1-

disregulering 'n potensiële bydraer is tot insulienweerstandigheid in die lewer wat deur spanning en inflammasie veroorsaak word.

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List of Abbreviations

ANOVA	Analysis of Variance
bp	Base pair
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CRP	C-Reactive protein
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
EF-1α1	Elongation factor-1 alpha 1
FBS	Fetal bovine serum
FOXO1	Forkhead box protein 1
F	Cortisol
FFA	Free fatty acids
G6Pase	Glucose 6-phosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GLUT	Glucose transporter
GSK3	Glycogen synthase kinase 3
G6P	Glucose 6-phosphate
Hsp90	Heat shock protein 90
IKKβ	Inhibitor of nuclear factor kappa B subunit beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IR	Insulin receptor

IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
PAI-1	Plasminogen activator inhibitor-1
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	Phosphoinositide 3-kinase
p-Akt	Phosphorylated Akt
p-FOXO1	Phosphorylated FOXO1
PKA	Protein Kinase A
qPCR	Quantitative PCR
RLU	Relative light units
RNA	Ribonucleic acid
T2DM	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TNFR	TNF Receptor
TNF-α	Tumour necrosis factor

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Chapter 1: Literature Review

1.1 Introduction

In 2019, the International Diabetes Federation (IDF) reported that approximately 463 million adults between the ages of 20 – 79 years have diabetes; this is expected to rise to 700 million adults by 2045[1]. Furthermore, approximately 1.8 million adults in South Africa were diagnosed with diabetes, with an estimated 1.5 million adults undiagnosed[1]. The increase in the prevalence of diabetes in South Africa could be attributed to a number of factors including economic shifts and urbanization associated with changes in nutrition and obesity[2]. In 2000, it was estimated that 87% of diabetes cases in South Africa were due to individuals being overweight or obese[3]. This is troubling since a systemic analysis in 2013 estimated that approximately 38% of men and 69% of women in South Africa were considered overweight or obese[4]. Diabetes is the second most common cause of death in South Africa, and seventh most common globally, which emphasizes the magnitude of this epidemic[1,5].

The development of insulin resistance, usually associated with type II diabetes mellitus (T2DM), can be linked to chronic inflammation. Chronic inflammation is generally referred to as inflammation that persists for a prolonged period of time, often months or even years[6]. This can lead to damage of the body's cells, tissues, and organs. Chronic inflammation is often considered to be low-level inflammation with subtle, if any, symptoms. Inflammation, whether acute or chronic, is associated with the stress response. Inflammation initiates the stress response through inflammatory mediators[7]. Stress is also a natural reaction to anything perceived as threatening, thereby associated with the fight-or-flight response. The activation of the stress response results in the synthesis and release of various biochemical mediators including glucocorticoids (GCs). Some of these mediators such as GCs function primarily to suppress inflammation present in the body[7,8]. The stress response subsides over a short period of time once the threat has been eliminated, allowing the stress levels of the organism to return to its normal function[9]. Chronic stress however, due to continuous exposure to a stressor, is often associated with the inability to sufficiently suppress inflammation in the body. This can lead to irreversible damage to tissues and organs[6,10]. Chronic stress is attributed to the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis leading to excessive cortisol (F) concentration in

circulation over time, which would subsequently result in the inability to suppress inflammation and the promotion of certain diseases[10,11]. Prolonged exposure to GCs induced by chronic exposure to a stressor may lead to several pathological conditions, such as hyperglycemia[12–14]. Furthermore, GC treatment for a number of inflammatory diseases also contribute to increasing excess circulating GC concentrations[15].The insulin signalling pathway is an intricate, multi-branched system that can be influenced at one or more levels due to several different factors. The pathway is largely controlled by insulin, which transduces its chemical signal by binding to the insulin receptor (IR) present in the plasma membrane of numerous cells. Binding of insulin to its cognate receptor triggers a phosphorylation cascade which includes protein kinase B (PKB), also referred to as Akt[16]. Akt is a central signalling protein in the insulin signalling pathway with phosphorylated Akt regulating a few sub-branches of the pathway, including the translocation of the glucose transporter 4 (GLUT4) in insulin responsive tissues, activation of glycogen synthesis, and inhibition of gluconeogenesis[17]. This ultimately reduces the rise in blood-glucose back to basal levels in individuals with a healthy functioning insulin signalling pathway allowing it to regulate glucose metabolism efficiently.

Gluconeogenesis is a metabolic pathway which occurs predominately in the liver during periods of fasting. It is responsible for *de novo* glucose production, thereby increasing blood-sugar concentration[18]. The rate of gluconeogenesis is predominantly controlled by the activity of unidirectional enzymes, namely phosphoenolpyruvate carboxykinase 1 (PEPCK), glucose-6-phosphatase (G6Pase), and fructose 1,6-bisphosphatase (FBPase)[19]. The main transcription factor responsible for transcribing *g6pc* and *pck1* genes is the forkhead box transcription factor 1, more commonly referred to as FOXO1[20]. Phosphorylated Akt is able to directly phosphorylate FOXO1 in the nucleus[21]. The phosphorylation of FOXO1 prevents the transcription factor from transcribing the *pck1* and *g6pc* genes necessary for gluconeogenesis[21,22]. Furthermore, the phosphorylation of FOXO1 causes the transcription factor to translocate to the cytoplasm of cells. Thus, gluconeogenesis is halted due to lack of key enzymes transcribed by FOXO1, resulting in the inhibition of *de novo* glucose production. There is great difficulty in managing insulin resistance due to the complexity of the pathway: dysregulation at one or several nodes of the signalling pathway could be responsible for a cell/tissue becoming resistant to insulin. The main

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contributor to the development of insulin resistance is the phosphoinositide-3-kinase (PI3K)/Akt pathway remaining largely inactive. Thus, Akt is not phosphorylated during insulin stimulation, and as a result is unable to inhibit FOXO1[23]. As a result, FOXO1 remains active in the nucleus and continues to promote glucose production, further contributing to high blood-glucose concentration[24]. The aim of this review is to discuss the insulin signalling pathway and the regulation of glucose metabolism. In addition, the development of insulin resistance and the mechanism thereof will be discussed in detail, including the role stress and inflammation play in its development. Finally, the role of the FOXO1 transcription factor in GC- and cytokine-induced insulin resistance will be discussed due to its crucial role in glucose metabolism.

1.2 Glucose homeostasis and the insulin signalling pathway

1.2.1 The role of glucose homeostasis in cellular functioning

The body requires a constant supply of energy to maintain its various functions and to keep its cells and organs active. One of the major energy sources is glucose, a monosaccharide carbohydrate. Glucose is the preferred energy source of the brain under physiological conditions, which utilizes approximately 20% of glucose-derived energy in the whole body[25,26]. Other major parts of the body that make use of glucose include adipose tissue and muscle cells to name a few[26]. It is therefore of great importance to maintain a continuous supply of glucose within the body – best described as glucose homeostasis.

Homeostasis is referred to as any self-regulating process by which biological systems tend to maintain stability while adjusting to conditions that are optimal for survival[27]. Glucose homeostasis must therefore keep the glucose concentration within a narrow range for the human body to have enough energy to function optimally. Glucose can come from three possible sources: absorption from the intestine following the consumption of a meal containing carbohydrates, glycogenolysis, a process in which stored glycogen is broken down into glucose, and gluconeogenesis, the production of glucose from precursors present in the body[28,29]. These processes are largely controlled by the liver.

Carbohydrates are broken down throughout the digestive system after the consumption of a meal and are absorbed as monosaccharides. This includes the absorption of glucose by the small intestine[29,30]. Glucose enters the enterocytes of the

small intestine *via* the sodium-dependent glucose co-transporter 1 (SGLT-1), which uses the electrochemical gradient of Na⁺ ions to move glucose across its membrane[31]. Intracellular glucose is released into the extracellular membrane near blood capillaries through GLUT2 *via* facilitated diffusion[31,32]. The rise in blood glucose concentration stimulates the secretion of insulin from the β-cells of the pancreas.

1.2.2 Production and secretion of insulin

The insulin molecule is a dipeptide containing A and B chains linked by disulphide bridges. The A chain is made up of 21 amino acids and the B chain has 30 amino acids, making this a dipeptide comprising of 51 amino acids in total[33]. Insulin is synthesized and secreted from the β-cells of the islets of Langerhans of the pancreas. The hormone is synthesized as a larger single precursor called preproinsulin[34]. It contains a signal peptide, the B-chain segment of insulin, a C-peptide, and the A-chain segment of insulin[34]. Once preproinsulin is synthesized in the cytosol, the signal peptide directs the precursor molecule to the endoplasmic reticulum (ER) for translocation. Whilst in the ER, the signal peptide is cleaved from the rest of the precursor, which assists in the formation of disulphide bonds and protein folding of the molecule known as proinsulin[34]. The signal peptide is then degraded while the newly folded proinsulin is transported to the Golgi apparatus in small vesicles. As these small vesicles move through the Golgi apparatus, they undergo a process of maturation in which the pH decreases from approximately 6.5 to about 5.5 in the mature secretory granule[35]. During this process, the C-peptide is cleaved from proinsulin to produce insulin.

The insulin hormone is secreted from the β-cells of the islets of Langerhans in response to glucose, various other monosaccharides, amino acids, and fatty acids. Glucose, however, is the primary stimulus and is required for normal functioning of energy metabolism[36,37]. The islets containing β-cells are surrounded by a dense network of capillaries that allows for an easy exchange of nutrients between the cells and circulatory system. As a result, β-cells can sense any changes in nutritional state quickly, as well as allow for the secretion and diffusion of insulin into circulation rapidly[36]. In order for β-cells to secrete insulin, the glucose concentration in the blood needs to increase to above 8 – 10mM to cause depolarization of the cells' membrane[33]. Glucose enters the β-cells through GLUT2 where it can enter the glycolytic pathway to produce adenosine triphosphate (ATP). This results in the closing of potassium

channels (K_{ATP} channels), that are sensitive to the levels of ATP within the cell, causing depolarization[38,39]. The depolarization results in the opening of voltage-gated membrane channels that allow calcium ions (Ca^{2+}) into the cell. The rise in intracellular Ca^{2+} concentration triggers insulin secretion[38,39]. The mature secretory granules containing insulin travel to the membrane and fuse with the membrane to release insulin *via* exocytosis[39].

It has been found that insulin is secreted in a 'bi-phasic' pattern; the first phase is short-lived lasting only a few minutes, followed by a sustained second phase. The first phase is a result of the immediate release of insulin from vesicles that are already found close to the β-cell membrane, docked and primed, whereas the second phase occurs due to the recruitment of the remainder of the insulin containing granules to the β-cell membrane and lasts as long as the elevation of blood-glucose is present[37,40].

1.2.3 Insulin Signalling Pathway: The PI3K/Akt branch

The insulin signalling pathway is an intricate, multi-branched system (Figure 1.1) that can be influenced on one or more levels due to several different factors. This pathway is largely controlled by the insulin hormone secreted from the pancreas. After the secretion of insulin into the blood, the hormone binds to the IR: a transmembrane protein belonging to the receptor tyrosine kinase family[16]. The IR consists of two α-subunits located on the outer cell surface and two transmembrane β-subunits. The subunits are joined by disulphide bonds[17]. The binding of insulin to the α-subunits causes the β-subunits to undergo a conformational change that activates the intrinsic kinase activity of the receptor. This allows the auto-phosphorylation of specific tyrosine residues present in the β-subunits, leading to the further activation of the kinase[17,41]. The phosphorylated tyrosine residues present in the intracellular part of the receptor recruit adaptor proteins such as the insulin receptor substrate (IRS). IRS has no intrinsic enzymatic activity, but rather acts as an 'adaptor' that assists in organizing signalling complexes. The IRS protein interacts with the phosphorylated IR through its phosphotyrosine binding (PTB) domain (Figure 1.2)[42,43]. In doing so, IRS becomes phosphorylated at its own tyrosine residues, which open up binding sites that can recruit other proteins to the signalling complex[43].
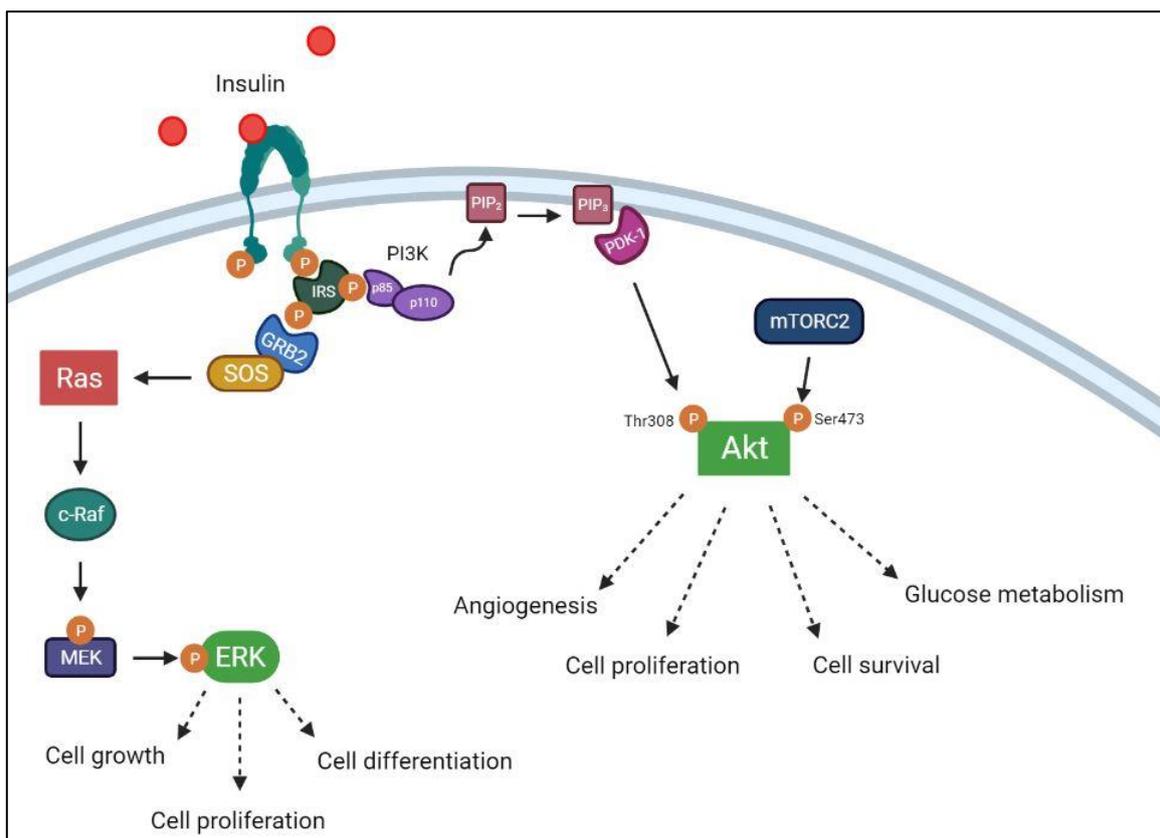
Figure 1.1. Schematic illustration of the insulin signalling pathway activated by insulin including some of the downstream targets and effects. Insulin binds to the IR resulting in the protein's activation. Following this, IRS is recruited and activated by IR. IRS can recruit proteins containing SH2 domains such as PI3K or GRB2. GRB2 goes on to activate the Ras dependent pathway, which is responsible for the activation of several transcription factors involved in cell growth, proliferation, and differentiation. The activation of the PI3K branch results in the activation of Akt, a protein involved in processes including angiogenesis, cell proliferation and survival, and glucose metabolism. Image adapted from Kumar, M. *et al.*[44], and Ceresa, B. *et al.*[45] Image drawn in Biorender.

There are several proteins that can bind to the phosphotyrosine residues of the IRS resulting in a signalling cascade as depicted in Figure 1.2 that regulates various branches of the insulin signalling pathway, such as gene expression, protein synthesis, glycolysis, and cell proliferation, to name a few[43]. One of the main signalling proteins to bind to the IRS is PI3K, which forms part of the PI3K/Akt pathway and influences glycolysis amongst other cellular processes. PI3K consists of a regulatory subunit, p85, and a catalytic subunit, p110[46]. The p85 subunit contains a Src-homology-2 (SH2) domain, allowing it to 'dock' onto the phosphorylated tyrosine residues of the IRS[47]. This results in the activation of p110, which rapidly phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP_3)[17]. PIP_2 and PIP_3 are membrane-bound phospholipids, where PIP_3 is the effector of downstream targets of the PI3K pathway[48].
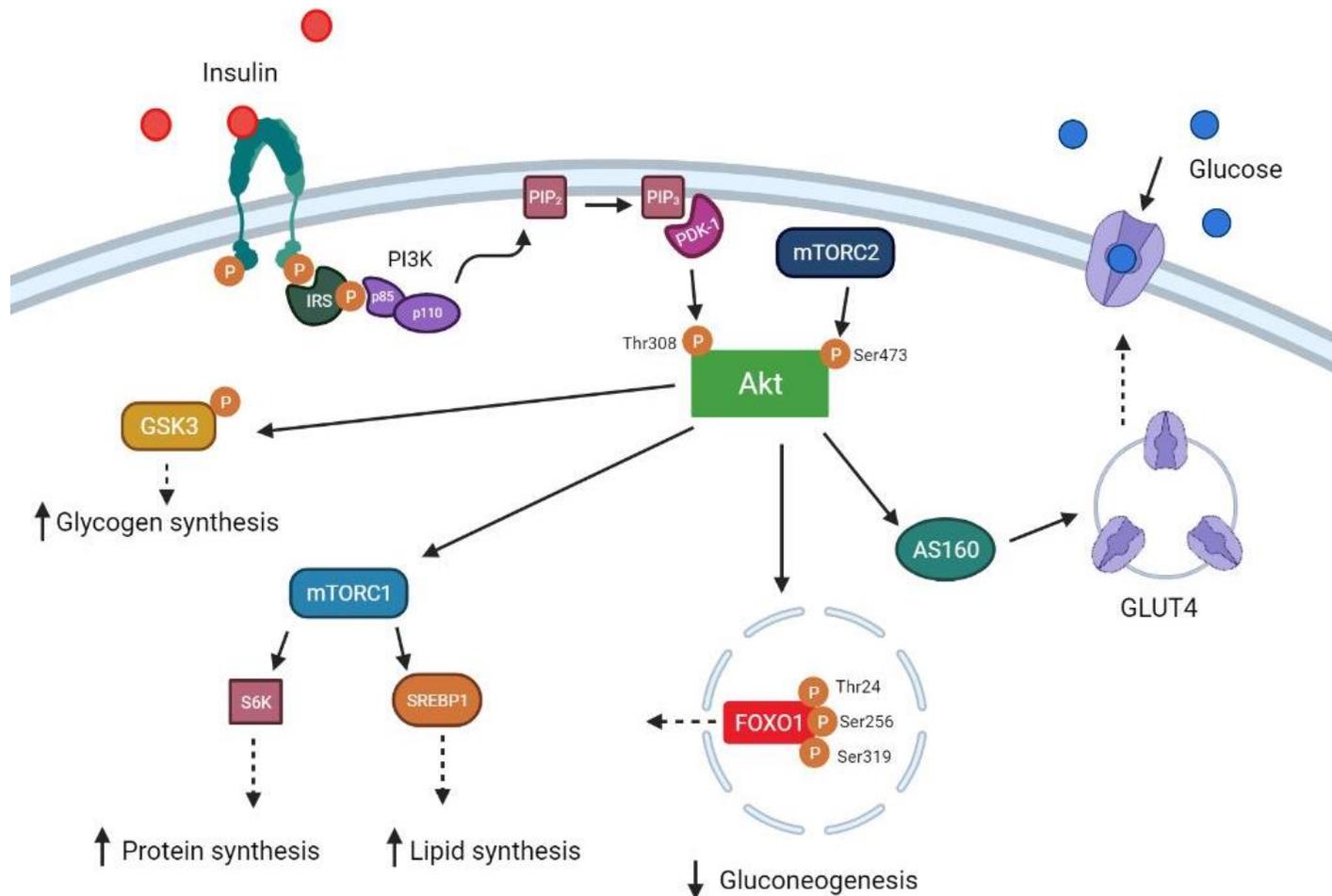
Figure 1.2. Schematic illustration of the activation of the PI3K/Akt pathway by insulin and the downstream targets of Akt. Insulin activates the IR upon binding, resulting in its activation and subsequent recruitment of IRS. IRS is activated by IR and recruits PI3K which catalyses the conversion of membrane bound PIP_2 to PIP_3. Following this, PDK1 is recruited along with Akt, where PDK-1 phosphorylates Akt at its Thr308 residue. Akt becomes fully activated once mTOR2 phosphorylates the protein at its Ser473 residue. Akt acts upon several downstream targets, including GSK3, mTORC1, FOXO1, and AS160, that results in the regulation of glucose, protein, and lipid metabolism, as well as glucose uptake. Glucose is transported by GLUT4 into adipose and muscle tissue. Image adapted from Kumar, M. *et al.*[44], and Boucher, J. *et al.*[17]. Image drawn in Biorender

The phosphorylation of PIP_2 to PIP_3 attracts proteins that contain pleckstrin homology (PH) domains, such as phosphoinositide-dependent kinase-1 (PDK-1) and Akt[48]. Upon binding to PIP_3, Akt undergoes a conformational change that exposes key phosphorylation sites[49,50]. PDK-1 is a serine/threonine kinase that is found to be active in the cytosol but is recruited to the membrane through its PH domain after PIP_2 to PIP_3 conversion takes place[51]. Whilst both Akt and PDK-1 are bound to the plasma membrane and is in close proximity to each other, PDK-1 phosphorylates Akt at its threonine 308 (Thr308) residue. Thus, through the presence of PH domains, the hormone signal is able to shift from the membrane into the cytosol[51].

Akt is a serine/threonine kinase that is a key mediator of many signal transduction processes and plays a role in regulating proliferation, apoptosis, and insulin signalling (Figure 1.2). In its active state, it regulates a myriad of substrates. In order for Akt to be activated fully, it requires phosphorylation of its Thr308 residue by PDK-1, as well as phosphorylation of its serine 473 (Ser473) residue[50]. The mechanism by which the Ser473 residue becomes phosphorylated has been controversial. It was originally assumed that PDK-1 is responsible for phosphorylating both Thr308 and Ser473, activating Akt[52]. It has since been established that PDK-1 does not directly phosphorylate Ser473 but may indirectly promote full activation[53]. In addition, it was suggested that after the initial phosphorylation of Thr308, Akt could auto-phosphorylate at its Ser473 residue[54]. Furthermore, integrin-linked kinase (ILK) has also been reported to phosphorylate Akt at Ser473[52,55], however it has since been suggested that it may only contribute indirectly to Akt phosphorylation at Ser473[56]. It is now largely thought that phosphorylation of Ser473 is modified by mammalian target of rapamycin complex 2 (mTORC2), also known as PDK-2[57,58]. mTOR is a conserved serine/threonine kinase that functions through one of two functional complexes, namely mTORC1 or mTORC2. mTORC2 contains several other subunits that are essential for various signalling pathways[59]. The fully activated Akt protein is now able to regulate numerous downstream events (Figure 1.2).

Firstly, Akt inhibits gluconeogenesis. It does this by regulating FOXO1 activity, which is a transcription factor responsible for the expression of key gluconeogenic genes. The inhibition of FOXO1 prevents the transcription of *pck1* and *g6pc*, genes coding for PEPCK and G6Pase enzymes respectively and is described in more detail in Section 1.3[60]. Without these enzymes, *de novo* synthesis of glucose is limited, which

would prevent blood glucose from increasing[61]. Furthermore, Akt is responsible for phosphorylation of the serine/threonine kinase, glycogen synthase kinase-3 (GSK3), inhibiting its ability to inactivate glycogen synthase (GS)[62,63]. In addition to this, the active Akt protein is involved in cellular glucose uptake from the bloodstream. Akt phosphorylates Akt substrate of 160 kDa (AS160), inhibiting its GTPase-activating protein activity[64]. This inhibition leads to the recruitment of GTP-bound AS160 target Rab proteins – Rab proteins make up a large group of proteins essential in vesicle formation and delivery, docking/fusion of vesicles to target membranes, and the recruitment of effectors[64,65]. The activation of AS160 target Rab proteins result in the trafficking of GLUT4-containing vesicles to the membrane of cells[65]. Here, the GLUT4-containing vesicles dock and fuse to the plasma membrane, allowing GLUT4 to facilitate the transport of glucose into the cell[66,67]. GLUT4 is primarily responsible for the influx of glucose into muscle and adipose cells. Glucose import into the liver however is slightly different and does not rely on GLUT4.

1.2.4 Insulin in the liver

Dietary glucose in the blood stream is taken to the liver *via* the hepatic portal vein[68]. Glucose travels through GLUT2 channels present as integral proteins in the plasma membrane of hepatocytes into the cytoplasm[69]. The secretion of insulin stimulated by increased blood glucose promotes glycolysis: the metabolism of glucose to pyruvate (Table 1.1). Insulin increases gene expression of glucokinase, which is responsible for the phosphorylation of glucose to glucose 6-phosphate (G6P), the first reaction of glycolysis as well as glycogen synthesis in the liver[70,71]. Furthermore, both insulin and glucose stimulate the dephosphorylation of the bi-functional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PFK2/FBPase2), promoting its kinase activity. The activation of 6PFK2/FBPase2 results in the increase of fructose-2,6-bisphosphate (F2,6P$_2$), a metabolite that activates the 6-phosphofructo-1-kinase enzyme (6PFK1). 6PFK1 is directly involved in generating fructose-1,6-bisphosphate (F1,6BP) from fructose-6-phosphate in the glycolysis pathway[70,72].

Insulin not only promotes glycolysis; it also stimulates the storage of excess glucose in the form of glycogen by activating GS. Insulin stimulates the dephosphorylation of GS, thereby activating the enzyme and allowing it to add a UDP-glucose molecule to a growing chain of glycogen[72,73]. Furthermore, the presence of G6P in the liver, which is influenced by the presence of insulin, is also an activator of GS by

dephosphorylating the enzyme[70,72]. The presence of G6P also dephosphorylates glycogen phosphorylase (GP), inhibiting the enzyme from shortening a glycogen chain by hydrolysing a glucose-1-phosphate (G1P) molecule (Table 1.1)[72].

Concurrent to the promotion of glycogenesis and glycolysis, insulin also inhibits gluconeogenesis (Table 1.1). Insulin inhibits the secretion of glucagon from α-cells of the pancreas[60,74]. In addition, as mentioned previously, insulin leads to the activation of Akt, which in turn phosphorylates FOXO1, inhibiting its ability to transcribe the gluconeogenic enzymes PEPCK and G6Pase[68,60]. Finally, insulin inhibits glycogenolysis by lowering the concentration of cyclic adenosine monophosphate (cAMP), resulting in phosphorylase kinase being unable to activate GP. GP is unable to break down glycogen to glucose as a result[75,76]. In promoting the rapid conversion of glucose in the various pathways mentioned above and preventing *de novo* glucose synthesis, glucose continually flows down a concentration gradient until blood glucose concentration is restored.

Table 1.1. Insulin's effects on glucose metabolism in the liver of healthy individuals.

Metabolic process	Insulin action	Mechanism	Reference
Glycolysis	Increases	Increase in glucokinase expression. Activating 6PFK1 *via* kinase activity of 6PFK2/FBPase2.	Guo *et al.*, and Massa *et al.*[70,71] Guo *et al.*, and Noguchi *et al.*[72,70]
Glycogenesis	Increases	Activating GS. Inhibiting GP *via* dephosphorylation. Inhibiting GSK3 *via* phosphorylation.	Noguchi *et al.*, and Bouskila *et al.*[72,73] Noguchi *et al.*[72] Lee *et al.*[77]
Gluconeogenesis	Inhibits	Inhibiting the transcription of gluconeogenic enzymes *via* inhibition of transcription factors. Inhibits secretion of glucagon from pancreatic α-cells.	Rea & James, and Hatting *et al.*[68,60] Hatting *et al.*[60]
Glycogenolysis	Inhibits	Inhibiting GP *via* dephosphorylation.	Noguchi *et al.*[72]

1.2.5 The role of glucagon and the production of glucose

Glucose homeostasis also encompasses mechanisms that increase glucose production when blood glucose drops below its normal threshold. A decrease in blood glucose triggers the peptide hormone glucagon to be secreted from the α-cells of the islets of Langerhans in the pancreas[78,79]. Glucagon secretion from the pancreas is triggered in a similar manner to that of insulin. The decrease in blood glucose is associated with a decrease in ATP. The ATP-sensitive K_{ATP} channels of the α-cells causes depolarization of the cell membrane due to the decrease in ATP, opening both voltage-dependant Na^+ and Ca^{2+} channels[78,80,81]. Much like the β-cells of the pancreas, the influx of Na^+ and Ca^{2+} results in glucagon secretion from the α-cells[78,80,81].

Glucagon is responsible for increasing hepatic glucose production through glycogenolysis and gluconeogenesis. Glucagon binds to its receptor (known as the glucagon receptor), a G protein-coupled receptor associated with the GTPase switch proteins $G_{\alpha s}$ and $G_{\alpha q}$[82,83]. The activation of $G_{\alpha s}$ results in increasing cAMP levels, subsequently leading to protein kinase A (PKA) activation[83]. Active PKA can alter glucose metabolism in several ways. Firstly, PKA activates phosphorylase kinase, which in turn activates GP, an enzyme that plays a role in glycogen breakdown to produce G6P. G6P can then be converted into glucose during glycogenolysis[83–86]. Secondly, PKA activates cAMP response element binding (CREB) protein, a transcription factor that plays a role in inducing the transcription of the gluconeogenic genes *pck1* and *g6pc*, ultimately increasing the rate of gluconeogenesis[83–86]. Finally, PKA activates the FBPase2 activity and inhibits the 6PFK2 kinase activity of the bi-directional enzyme 6PFK2/FBPase2. As a result, $F2,6P_2$ concentration decreases, leading to the inhibition of 6PFK1 activity and the activation of FBPase which is responsible for the production of F6P in gluconeogenesis[83,85,86]. The activation of $G_{\alpha q}$ activates phospholipase C that ultimately results in an increase in intracellular Ca^{2+}. Ca^{2+} are well known secondary messengers that play a role in the activation of several proteins, including promoting the activation of nuclear FOXO1 in gluconeogenesis, and indirectly inhibiting GS to prevent glycogen production[83,87–90].

Taken together, blood glucose is tightly regulated to ensure cells of the body have an adequate energy supply. Dysregulation in glucose homeostasis can as a result, have serious consequences including cells becoming resistant to insulin, which is relevant to the current study.

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1.2.6 Insulin resistance

Insulin resistance is simply defined as a decrease in cellular or tissue responsiveness to normal circulating insulin concentration, thereby resulting in an attenuated biological response[91,92]. In healthy individuals, insulin promotes glucose uptake from the blood into mainly skeletal muscle and white adipose tissue, while inhibiting glucose production in the liver[93–95]. In insulin resistant states, target tissues do not respond to normal insulin concentration, resulting in a reduction in glucose uptake by these tissues. The elevated blood glucose concentration triggers a further increase in insulin secretion from the β-cells of the pancreas in an attempt to return to ideal glucose homeostasis[93–95]. The increased demand placed on β-cells to secrete insulin eventually leads to its dysfunction, whereby hyperglycaemia is evident in patients[93–96]. The manifestation of insulin resistance, however, is complex and not wholly understood. It is however, well known that obesity plays a major role in insulin resistance. Obesity is characterized by excessive growth of adipose tissue, resulting from an increase in adipocyte number (referred to as hyperplasia)[97–99]. Several studies have shown the positive correlation between obesity and insulin resistance risk[98,100–102]. Additionally, there are several obesity-associated factors that have been implicated in the development of insulin resistance. Firstly, obesity is often associated with chronic, low-grade inflammation. The inflammatory mediators tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) have been implicated in the development of insulin resistance, as both have been found to be elevated in patients with insulin resistance[103–105]. Secondly, obesity causes an increase in the rate of lipolysis due to hyperplasia, resulting in a persistent increase in free fatty acid (FFA) concentration in circulation. FFAs inhibit insulin-stimulated glucose uptake, thereby dampening insulin sensitivity of tissues[106–109]. Finally, both adipokines that are secreted from adipocytes and FAs can result in a build-up of reactive oxygen species (ROS) that can lead to oxidative stress. ROS and oxidative stress have been implicated in the development of insulin resistance with antioxidant treatment, both *in vitro* and *in vivo*, shown to improve insulin sensitivity[110–112,113,114]. Increased F secretion has also been linked with obesity, although the exact cause and mechanism in which this occurs is unclear. Some studies suggest psychological stress associated with obesity may be the reason for elevated F levels[115–118]. Other studies associate increased stress and elevated F with weight gain[119–121]. Furthermore, some research shows that dysregulation in the

metabolism of F *via* the enzyme 11-β-hydroxysteroid dehydrogenase (11β-HSD1) results in hyperactivity of the HPA and increased F secretion[122–124]. It is, however, evident that F remains elevated in obese patients.

Insulin resistance is further associated with dysregulation of the mechanisms that maintain glucose homeostasis. Firstly, the glycolytic pathway is slowed down due to decreased glucose transport into insulin responsive tissues, and/or a decrease in activity of enzymes in glycolysis that require insulin activation, such as GK, and changes in the levels of metabolites required, such as $F2,6P_2$[70,125,126]. Furthermore, research shows that GS activation is hindered in insulin resistance, causing the rate of glycogenesis to decrease[127–130]. In addition, it has been found that elevated circulating FFAs in obese, insulin resistant patients are able to hinder the ability of insulin to suppress glycogenolysis[131–134]. Two studies have shown that FFAs increase the activity of the serine/threonine kinases, protein kinase C (PKC) and IκB kinase (IKK), which could result in the decrease in IRS phosphorylation and therefore contribute to insulin being unable to inhibit glycogenolysis[135,136]. Finally, gluconeogenesis is not sufficiently halted in insulin resistance, resulting in glucose output despite the presence of insulin[60,137]. Several studies have found a correlation between insulin resistance and increased expression of the gluconeogenic transcription factor, FOXO1, and the genes it transcribes, namely *pck1* and *g6pc*[60,138,139]. Furthermore, deletion of *foxo1* appears to improve insulin sensitivity[140,141]. For this reason, the FOXO1 transcription factor is of great interest to this study.

1.3 The role of FOXO1

1.3.1 Structure and function

The forkhead box (FOX) transcription factor family is characterized by a highly conserved forkhead domain[142]. FOX proteins are made up of four domains: a forkhead DNA binding domain, a nuclear localization signal, a nuclear export sequence, and a transactivation domain (Figure 1.3)[143].The O-class subfamily of forkhead box transcription factors (FOXOs) found in mammalian cells consist of four members, namely: FOXO1, FOXO3a, FOXO4, and FOXO6[142]. The expression of the various FOXO proteins differ across all tissues, with FOXO1 and FOXO3a being present in nearly all mammalian tissues[144].

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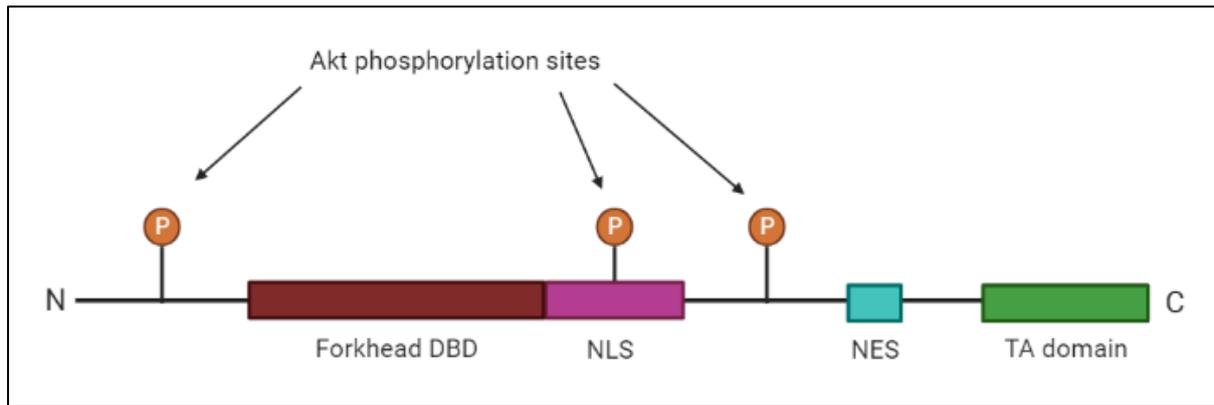
Figure 1.3. Schematic representation of the structure of FOXO proteins. All FOXO proteins contain a forkhead DNA binding domain (DBD), a nuclear localization signal (NES), a nuclear export sequence (NES), and a transactivation (TA) domain. The sites at which Akt can phosphorylate FOXO are shown. Image drawn in Biorender.

FOXO1 is a protein with several functions including the regulation of apoptosis, differentiation, proliferation, and metabolism to name a few (Table 1.2)[145]. FOXO1 promotes apoptosis when active in the nucleus[146]. Furthermore, FOXO1 negatively regulates the differentiation of a number of cells including adipocytes and pancreatic cells[147,148]. FOXO1 also plays a role in the regulation of metabolism by promoting hepatic glucose production *via* the enzymes G6Pase and PEPCK[20].

Table 1.2. Genes regulated by the FOXO1 transcription factor.

Gene	Protein	FOXO1 Mechanism	Function	References
pck1	PEPCK	Increases	Gluconeogenesis	van der Vos & Coffer[149], and Nakae *et al.*[150]
g6pc	G6Pase	Increases	Gluconeogenesis	van der Vos & Coffer[149]. and Nakae *et al.*[150]
bim	BIM	Increases	Apoptosis	Fu, & Tindall[151], Gilley *et al.*[152], and Glauser & Schlegel[153].
fasL	Fas ligand	Increases	Apoptosis	Fu & Tindall[151], and Glauser & Schlegel.[153]
cdkn1b	Cyclin-dependent kinase inhibitor 1B (p27)	Increases	Cell cycle arrest	Glauser & Schlegel.[153], and Jiang *et al.*[154]
gadd45	Growth arrest and DNA damage-inducible protein 45	Increases	Cell cycle arrest	Furukawa-Hibi *et al.*[155]

FOXO1 can regulate the transcription of a gene in several ways, as depicted in Figure 1.4. Firstly, FOXO1 can bind directly to the promoter of a target gene (Figure 1.4A), followed by the recruitment of a co-activator to initiate gene transcription. For example, peroxisome proliferative activated receptor-gamma co-activator 1 (PGC-1α) co-activates FOXO1 in order to begin transcription of *pck1* and *g6pc* for gluconeogenesis[85,156,157]. FOXO1 can also act as a co-activator to other transcription factors, such as binding to the Ets-1 transcription factor to promote angiogenesis[158,159] (Figure 1.4B). In addition, FOXO1 can repress gene transcription by preventing other transcription factors from binding to the promoter (Figure 1.4C). For example, FOXO1 is able to bind to the promoter of the sterol regulatory element-binding protein 1 (srebf1) gene instead of liver X receptor α (LXRα) in fasted states, preventing the transcription of SREBP-1, a protein involved in lipogenesis[160,161]. Finally, FOXO1 can bind to a gene promoter along with another transcription factor in a cooperative manner resulting in upregulation of transcription (Figure 1.4D). For example, Runt-related transcription factor 3 (RUNX3) and FOXO1 bind to their respective elements on the promoter of the *bim* gene, resulting in an increase in the pro-apoptotic protein[162,163].
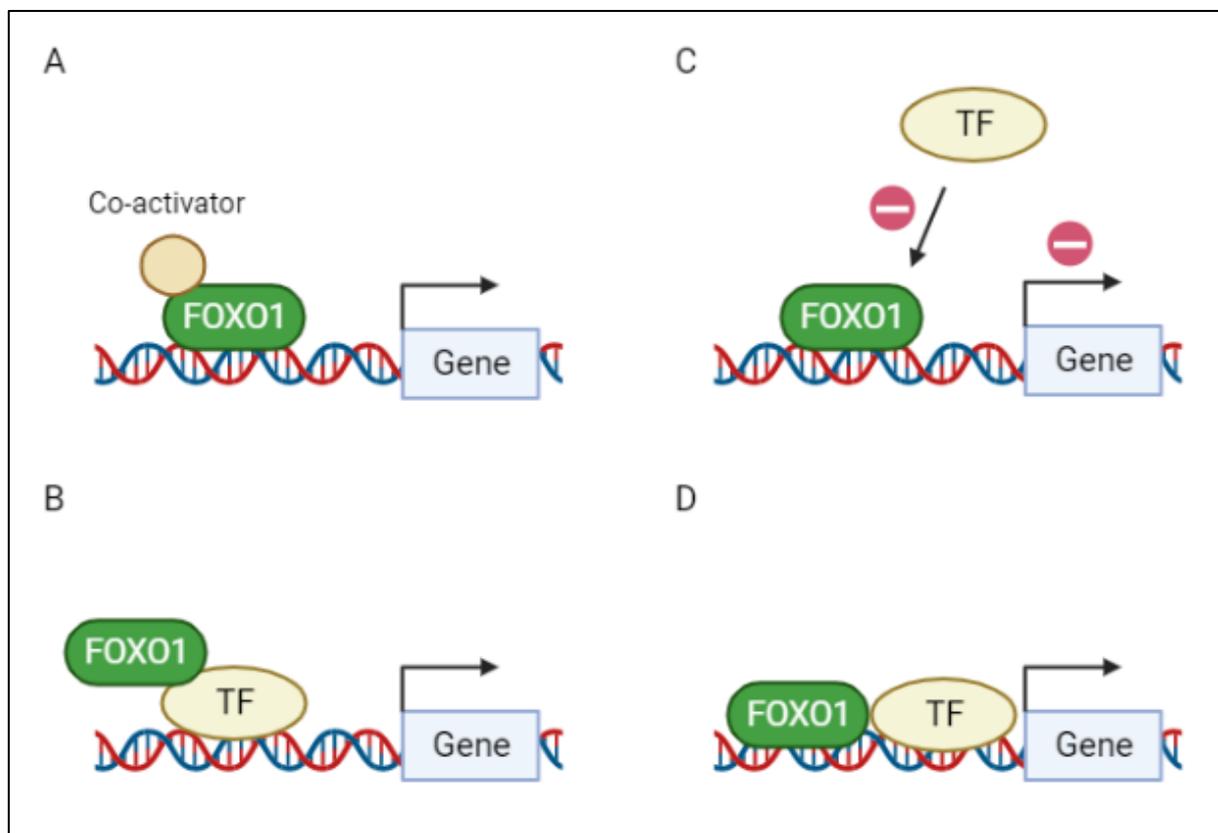
(Figure legend on next page).

Figure 1.4. Schematic illustration of different mechanisms of FOXO1 transcriptional regulation. FOXO1 can recruit co-activators to increase transcription of genes **(A)**). In addition to this, FOXO1 itself can act as co-activator to other transcription factors (TF) to regulate gene transcription **(B)**. FOXO1 can compete with other transcription factors in binding to a promoter, repressing gene transcription **(C)**. FOXO1 is also able to bind co-operatively with other transcription factors to regulate gene transcription **(D)**. Image adapted from Glauser, D. A and Schlegel, W[153]. Image drawn in Biorender.

FOXO1 is mainly situated in the nucleus where it can regulate the transcription of G6Pase and PEPCK responsible for *de novo* glucose synthesis. As described previously, gluconeogenesis is active during periods of fasting, whereby glucose is produced in response to the decrease in blood-glucose concentration. The rate of gluconeogenesis is predominantly controlled by the activity of unidirectional enzymes, PEPCK, FBPase, and G6Pase. As blood-glucose concentration decreases, the gluconeogenesis pathway is activated indirectly by FOXO1. FOXO1 plays a major role in the transcriptional activation of *g6pc* and *pck1* through its association with the promoters of these gluconeogenic genes[144,164]. Consequently, this leads to an increase in G6Pase and PEPCK protein levels that play a role in irreversible reactions of gluconeogenesis[19].Studies have shown that overexpression of both PEPCK and G6Pase increase hepatic glucose production[165–167]. FOXO1 and its role in energy metabolism is therefore of particular interest to the current study and its regulation by insulin will be discussed next in more detail.

1.3.2 Regulation of FOXO1 in the insulin signalling pathway

While the expression of PEPCK and G6Pase in glucose metabolism have been found to increase gluconeogenesis as mentioned above, the activity and expression of FOXO1 in glucose metabolism has also been shown to play a role. Several studies have shown that knockdown of FOXO1 in liver cells results in a decrease in PEPCK and G6Pase expression, as well as a reduction in hepatic glucose production[168–170]. In addition to this, repressing *foxo1* has improved insulin sensitivity in insulin resistant phenotypes[150,168]. Furthermore, increased expression of FOXO1 has been observed in insulin resistance and diabetic patients, correlating to the increase in glucose production[150,171]. Due to the diverse functionality of FOXO1, the transcription factor is tightly regulated at a transcriptional and post-translational level. While the regulation on the promoter of *foxo1* is largely unknown, several factors have been found to influence *foxo1* expression[172–176]. In addition, the activity of FOXO1 can be regulated by several post-translational modifications, including phosphorylation, acetylation,

ubiquitination, and methylation[142,177,178]. These regulatory mechanisms alter the transcriptional activity of FOXO1 and for this reason will be investigated further.

1.3.3 Nuclear export of FOXO1

Upon the consumption of a meal and the consequent rise in blood-glucose levels, insulin is secreted from the β-cells[16]. The binding of insulin to the IR results in a phosphorylation cascade involving Akt as described earlier (Section 1.2).

Phosphorylated Akt is responsible for directly phosphorylating nuclear FOXO1 at three residues, namely Thr24, Ser256, and Ser319, which are conserved across the forkhead family[16,21] (Figure 1.5). The phosphorylation of Thr24 and Ser256 functions to recruit and bind 14-3-3 proteins. The binding of 14-3-3 proteins masks nuclear localization signals that are found on the DNA-binding domain, which inhibits the ability of FOXO1 to bind to DNA and limits any interaction with other transcription factors found in the nucleus. The 14-3-3 proteins also prevent FOXO1 from shuttling back into the nucleus[179,180]. The phosphorylation of Ser319 by Akt is followed by the phosphorylation of nearby residues Ser322 and Ser325 by CK1 (casein kinase 1). Furthermore, Ser329 is also phosphorylated by DYRK1A (dual specificity tyrosine-phosphorylation-regulated kinase 1A). These phosphorylated residues enhance a nearby nuclear export sequence located in the DNA-binding domain of FOXO1. It does this by signalling Ran (Ras-related nuclear protein), a nuclear transport protein, that further summons CRM-1 (Chromosomal maintenance 1), which results in nuclear export[179,180]. Nuclear export of FOXO1 ultimately hinders the transcription factor from being functional.

1.3.4 Degradation of FOXO1

Phosphorylation of FOXO1 also leads to its degradation *via* the ubiquitin-proteasome system (UPS). Before FOXO1 is recognized by the proteasome and targeted for degradation, ubiquitin needs to be transferred and assigned to substrates through the activation of three enzymes, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2 or UBC), and ubiquitin ligase (E3)[181]. It is believed that E3 recognizes the phosphorylation of FOXO1 and targets the protein for degradation[181,182]. E3 ligases are categorized into two main classes: RING-finger proteins or HECT-domain proteins. The E3 ligase that is thought to bind to FOXO1 is a multi-subunit RING-finger protein called SKP1-CUL1-F-box protein (SCF) complex[181]. The F-box

protein of the SCF complex is what determines substrate specificity. The function of the F-box protein is to bring SKP1, an adapter protein, into close proximity to the substrate[181,183]. SKP2 is the F-box protein that binds to FOXO1 by recognizing the Ser256 phosphorylation and brings the SCF complex into proximity[184] (Figure 1.5). Rbx1, the RING-finger protein of the SCF complex, recruits E2 to the complex. E1 activates ubiquitin molecules, a step requiring ATP, and then transfers the activated ubiquitin molecule to E2 *via* a thioester bond[185]. E2 is then able to transfer the ubiquitin protein onto the lysine residue-rich region of FOXO1[181]. This cycle of ubiquitin activation and transfer from E1 to P-FOXO1 occurs until a polyubiquitin chain of 4-5 residues is present on FOXO1 and can be recognized by the 26S proteasome. The proteasome hydrolyses the ubiquitinated P-FOXO1 into small peptides and amino acids and releases this back into the cell along with the ubiquitin molecules[186].
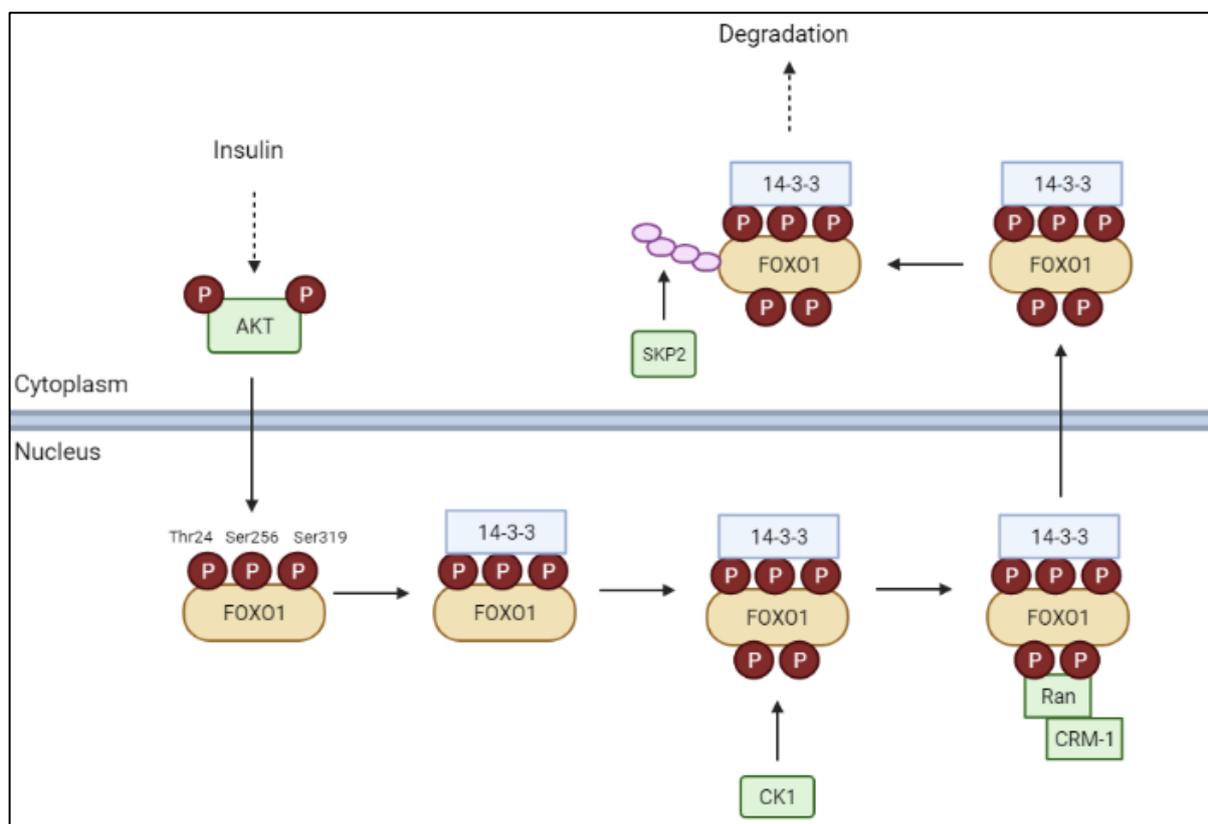
Figure 1.5. The phosphorylation and subsequent nuclear export and degradation of FOXO1. Insulin-activated Akt enters the nucleus and phosphorylates FOXO1 at three residues, namely Thr24, Ser256, and Ser319. Thr24 and Ser256 recruit 14-3-3 proteins and in the process inhibit FOXO1's DNA binding ability. Ser319 phosphorylation signals CK1 to further phosphorylate FOXO1 at Ser322 and Ser325, resulting in the recruitment of Ran and CRM-1 that drive nuclear export. Once in the cytoplasm, P-FOXO1 is recognized by SKP2 of the SCF complex, followed by the recruitment of the ubiquitination enzymes that ubiquinate P-FOXO1 and marking the protein for degradation *via* the 26S proteasome. Image adapted from Daitoku *et al.*[187], and Jiramongkol *et al.*[188]. Image drawn in Biorender.

1.3.5 Other post-translational mechanisms involved in regulating FOXO1

Besides phosphorylation and ubiquitination, FOXO1 is regulated by other post-translational modifications including acetylation. Several studies have shown that cAMP-response element-binding protein (CBP) and p300 (CBP/p300) directly acetylates FOXO1 at three of its lysine residues, namely K242, K245, and K262[142,187,189]. In doing so, acetylated FOXO1's DNA binding ability is attenuated, limiting transcription[142,177,187,189]. In addition, acetylation of FOXO1 increases its sensitivity for Akt phosphorylation[142,189]. Acetylation of FOXO1 has also been shown to be reversible, where Sirtuin 1 (Sirt1), a NAD-dependent deacetylase, binds to FOXO1 and deacetylates the transcription factor at K242, K245, and K262[142,177,190]. Whilst acetylation of FOXO1 inhibits its transcriptional activity, methylation of FOXO1 has a contradictory effect. FOXO1 can be methylated at arginine residues, Arg248 and Arg250, by protein arginine methyltransferase 1 (PRMT1). Methylation of FOXO1 prevents its phosphorylation by Akt and its subsequent movement into the cytoplasm[191–194]. Inhibition of PRMT1 has shown to prevent methylation of FOXO1 and as a result promote FOXO1 nuclear exclusion followed by its degradation[192,194].

1.3.6 Transcriptional regulation of *foxo1*

The mechanisms involved in the transcription of the *foxo1* gene remains largely unknown, despite its key role as a transcription factor. Evidence does exist however, which describes FOXO1 mRNA and protein expression to increase in the liver during a fasting state[173,195,196]. This supports the knowledge that gluconeogenesis is active in states of fasting. The transcription factors that play a role in the increase of *foxo1* gene expression during fasting states have not been extensively researched. A study by Arcidiacono *et al.,* looked at the effects the high-mobility group A1 (HMGA1), a mediator that plays a role in the regulation of genes for insulin and the IR, had on *foxo1* expression. Both FOXO1 mRNA and protein levels increase when HMGA1 is overexpressed, while silencing HMGA1 results in a decrease in FOXO1 expression, both at mRNA and protein level[176]. Another study by Wondisford *et al.,* wanted to determine whether glucagon could increase *foxo1* expression *via* CREB co-activators (CBP/p300), as they are known to play a role in transcription of *pck1* and *g6pc* along with FOXO1[173,197,198]. Interestingly, knockdown of the CREB transcription factor and p300 lowers FOXO1 mRNA, but not the depletion of CBP. Furthermore, three cAMP response elements (CREs) have been identified in the promoter of *foxo1*, suggesting

that p300 interacts with CREB that illicit its effects on these CREs[173]. In addition, the *foxo1* proximal promoter contains glucocorticoid response elements (GREs) allowing *foxo1* gene expression to be increased in response to GC treatment[174]. Qin *et al.* demonstrated that the deletion of these GREs inhibits Dex-induced *foxo1* expression[174].

While the post-translational modifications of FOXO1 are fairly well understood, the mechanisms that moderate the transcription of *foxo1* remain somewhat elusive. FOXO1's role in insulin signalling is vital and its dysregulation in insulin resistance evident. It is therefore of interest to determine factors that contribute to insulin resistance, such as stress and inflammation, and whether these factors affect FOXO1 expression and activity.

1.4 The role of inflammation in insulin signalling

1.4.1 Inflammation

Inflammation is commonly described as the immune system's response to harmful stimuli such as pathogens, cells that are damaged, compounds that are toxic, or irradiation[199]. The primary function of inflammation is to stimulate various inflammatory pathways that resolve or repair the damage done by harmful stimuli so that homeostasis is restored[199,200]. Many of the cells involved in the immune response secrete cytokines that induce a wide range of effects such as: signalling other cells involved in the immune system to move to the site of infection, and activating immune cells at the site of infection[201]. Ideally, the inflammatory response should be rapid, specific, and self-limiting[200]. Occasionally, infectious organisms and other agents resist host defences for an extended period, resulting in chronic inflammation. Chronic inflammation can also be a result of long-term exposure to irritants or foreign materials the body cannot eliminate, or autoimmune diseases[202]. Chronic inflammation could lead to a number of serious consequences, including tissue damage and degeneration, constitutively active immune cells that further modulate the immune response, and altering the normal functioning of tissues[203]. As a result, chronic inflammation is linked to many diseases such as cancer, asthma, rheumatoid arthritis, and insulin resistance[202,203,204]. The following section will discuss how inflammation may induce insulin resistance.

1.4.2 The mediation of the inflammatory response

As mentioned above, inflammation involves the delivery of various mediators of the immune system to the sites of infection and/or damage. Inflammation can be a result of both infectious and non-infectious factors (Table 1.3)[199]. The process of inflammation begins with localized inflammatory cells, such as mast cells, dendritic cells, and macrophages, at the site of infection[205]. Inflammatory cells secrete various cytokines and chemokines that signal for an increase in blood flow to the site of infection, bringing plasma proteins, neutrophils, and phagocytes to the site of infection[201]. The pro-inflammatory cytokines then activate neutrophils at the site of infection, allowing these cells to fight off the cause of the infection[205,206]. If the acute inflammatory response is unable to eliminate the cause of infection, the inflammatory process persists and resorts to additional measures[205]. These new characteristics include recruiting additional macrophages and leukocytes in place of neutrophils, and T cells [205]. A chronic inflammatory state could result however, if the influence of these immune cells combined are unable to fight the infection[205].

Table 1.3. Factors that result in an inflammatory response.

Non-infectious factors		
Physical	Burn, physical injury, foreign bodies, trauma	Stankov, and Chen *et al.*[199,207]
Chemical	Glucose, fatty acids, toxins, alcohol, chemical irritants	Chen *et al*, Hwi *et al.*, Rom & David, and Sears & Perry[107,199,208,209]
Psychological	Excitement, depression, anxiety, emotional stress	Chen *et al.*, and Furman *et al.*[199,210]
Infectious factors		
Pathogens	Bacteria, viruses, other microorganisms	Chen *et al.*, Mitchell & Isberg, and Porta *et al.*[199,211,212]

It should be noted that the inflammatory response described above is characteristic of infections. Chronic inflammation resulting from obesity, however, is characterised by

continuous activation of the immune response, without a causal factor such as a bacterium, or tissue damage[213]. Understanding chronic inflammation and its role in the manifestation of diseases is becoming increasingly important.

1.4.3 Chronic vs. acute inflammation

Inflammation can be separated into two broad categories: acute phase and chronic phase[214]. The acute phase response can be described by an increase in blood flow, accumulation of fluid, the arrival of leukocytes, fibroblasts, macrophages, and aggregation of platelets at the site of injury[214,9,215]. The activation of these cells in the initial immune response are able to release acute phase proteins (APPs) and cytokines at the site of injury[9,215]. Cytokines act as signals to mediate the inflammatory response, assist in activation of lymphocytes, and induce the expression of molecules required for the humoral immune response[216]. Chronic inflammation refers to inflammation that lasts for prolonged periods of time. Chronic inflammation can result from infectious organisms and other agents that resist host defences for an extended period, long-term exposure to irritants or foreign materials the body cannot eliminate, or autoimmune diseases such as rheumatoid arthritis[202]. The chronic phase may share many features with the acute phase, but due to its persistence it could lead to tissue degeneration, tissue damage, constitutively active immune cells that continuously produce cytokines that further modulate the inflammatory response and altering the normal functioning of tissues[203]. Interestingly, chronic inflammation has recently been identified as the potential link between obesity and insulin resistance[217,218]. A key characteristic of obesity is the presence of chronic low-grade inflammation. Up until recently, the exact mechanism of how obesity initiates the inflammatory response and causes chronic inflammation has not been completely understood[217–219]. It is therefore important to investigate emerging evidence on the potential ways chronic inflammation could result in the development of insulin resistance.

1.4.4 Chronic inflammation in insulin resistance

Physiological processes generally operate within a certain range of conditions and are maintained through mechanisms that aim to achieve homeostasis[220,221]. For example, glucose metabolism (Section 1.2) is maintained through mechanisms such as glycogenolysis and gluconeogenesis[28]. Inflammation is the body's protective response to influences that challenge homeostasis[220]. Inflammatory signals, such as cytokines and chemokines, can induce changes in specified biological mechanisms (i.e.,

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inhibition, activation, or suppression) in an attempt to restore homeostasis[220,221]. The persistence of this inflammation however, can result in changes in the 'range' of homeostatic points, causing the body to enter a pathological state[220]. A prime example is the correlation between chronic inflammation and metabolic syndrome. Metabolic syndrome encompasses glucose intolerance, obesity, high cholesterol, and hypertension[213]. The link between obesity and inflammation relates to the dysfunction of adipocytes in the human body. Adipocytes have recently been found to have toll-like receptors (TLRs)[213,222]. TLRs are receptors that are known to be present on cells of the immune system such as macrophages, B cells, mast cells, and T cells to name a few[222]. The function of TLRs in immunity is to recognise conserved parts of foreign invaders such as bacteria, parasites, fungi, or viruses, commonly referred to as pathogen-associated molecular patterns (PAMPs)[222,223]. PAMPs that are recognized by TLRs include lipopolysaccharides, lipoproteins, proteins, and nucleic acids[222,223]. Upon recognition by cells of the immune system, TLRs activate signalling pathways that produce cytokines, chemokines, or interferons, which brings about the inflammatory response[222]. Interestingly, TLRs on adipocytes are able to recognize dietary fatty acids present, activating the signalling pathways that result in the production of inflammatory mediators[222,224,225]. Studies show that TLR deficient mice had significantly reduced cytokine serum levels compared to wildtype mice[226,227]. The cytokines secreted by adipocytes, including TNF-α and IL-6, happen to be pro-inflammatory and thus contribute to the presence of inflammation. An increase in adipose tissue resulting from obesity further reinforces this inflammatory response due to the increase in production of these pro-inflammatory cytokines[213,225]. This could explain why the term low-grade chronic inflammation is commonly associated with obesity. Cytokines present in inflammation can directly regulate parts of the insulin signalling pathway through the activation of inflammatory pathways[217,218]. While acute inflammation results in temporary regulation of insulin signalling, chronic inflammation alters the functioning of insulin signalling indefinitely[228]. The persistent production of pro-inflammatory cytokines in chronic inflammation is of particular interest – elevated cytokine levels such as TNF-α and IL-6 are often elevated in insulin resistant states.

1.4.5 The role of cytokines in insulin resistance

Cytokines are small proteins that are secreted by cells as a method of interacting and communicating between cells[229]. Cytokines are able to regulate a number of processes

including both innate and adaptive immunity, inflammation, cell growth, cell differentiation, and cell death to name a few[229]. Cytokines involved in the immune response can be broadly categorized as either pro-inflammatory, or anti-inflammatory (Table 1.4)[230]. While cytokines are usually associated with inflammation due to injury, several studies have shown that other cells that do not form part of the immune system are able to secrete cytokines, particularly adipocytes, as mentioned previously[231–234]. Furthermore, cytokines can have functions outside of the immune response such as regulating the activity of transcription factors, liver development and regeneration, and modifying insulin signalling[34,237]. The mechanisms in which cytokines can regulate insulin signalling, specifically TNF-α and IL-6, will be discussed in detail due to their increased levels in insulin resistant patients[238–242].

Table 1.4. Cytokines secreted from cells of the immune system that have been implicated in insulin resistance and T2DM.

Cytokine	Source	Target	Effect on insulin signalling	References
TNF-α	Macrophages, mast cells, neutrophils, natural killer cells	Production and activation of B and T cells, stimulates productions of other cytokines, recruits immune cells to site of infection.	Inhibits activation of IRS by activating IKK and/or JNK pathway, increases the production of FFAs that activate kinases involved in insulin signalling.	Brotas *et al.*, Silva *et al.*, Horiuchi *et al.*, and Iqbal *et al.*[243–246]
IL-6	Fibroblasts, macrophages, T-cells, mast cells	Stimulates production of APPs, B cell maturation, antibody production.	Inhibits activation of IRS via JAK/STAT pathway.	Tanaka *et al.*, Velazquez-Salinas *et al.*, and Mauer *et al.*[247–249]
IL-1β	Monocytes, macrophages, dendritic cells	Promotes expression of various chemokines and cytokines, recruits cells to site of infection.	Inhibits IRS and inhibits phosphorylation of Akt via ERK pathway.	Eder, C., Wieser *et al.*, and Jager *et al.*[241,250,251]

1.4.5.1 TNF-α in insulin signalling

TNF-α is a well-known pro-inflammatory cytokine secreted mainly by macrophages. Specifically, adipose tissue macrophages are the primary source of TNF-α secretion[233,252,253]. TNF-α is the first cytokine to have been linked to insulin resistance[254]. There are several possible mechanisms in which TNF-α could affect insulin signalling discussed below.

Firstly, TNF-α can elicit a response through two receptors: TNF receptor-1 (TNFR-1, also known as p55) and TNF receptor-2 (TNFR-2, also known as p75)[255,256]. TNFR-1 is ubiquitously expressed, and therefore appears to be the key mediator of TNF-α signalling (Figure 1.6)[257]. The binding of TNF-α to TNFR-1 results in the intracellular domain of the receptor becoming exposed[257]. This leads to the recruitment of TNFR-1 associated death domain protein (TRADD) to the receptor complex[257,258]. TRADD acts as a scaffold protein that recruits other proteins to the complex: Fas-Associated Death Domain (FADD), TNFR Associated Factor 2 (TRAF2), and Receptor Interacting Protein (RIP)[257,258]. In the absence of inflammation, nuclear factor κB (NF-κB) can be found in the cytoplasm of cells under the control of Inhibitor of NF-κB (IκB) protein. In order for NF-κB to become transcriptionally active in the nucleus, IκB must release NF-κB[257,258]. For this to occur, TRAF2 recruits IκB kinase (IKK) to the protein complex on TNFR-1, where RIP activates IKK. Activated IKK can phosphorylate IκB, leading to its dissociation from NF-κB and target it for degradation[257–259]. Active NF-κB is a transcription factor involved in the production of a number of pro-inflammatory markers and cytokines that contribute to increased inflammation, such as C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), IL-6, and IL-1[260]. TNF-α signalling has been shown to inhibit insulin signalling. TNF-α-activated IKK can phosphorylate IRS-1 at several serine residues, namely Ser265, Ser302, Ser325, Ser336, Ser358, Ser407, and Ser408, thereby inhibiting any downstream signalling[254,261]. Furthermore, inhibition of IKK results in improved insulin sensitivity in the liver[262,263,264]. Inhibiting TNF-α signalling leads to an increase in insulin-induced tyrosine phosphorylation of IRS-1, increasing insulin sensitivity[265,266]. Furthermore, NF-κB inhibition in mice reduced insulin resistance and lipid abnormalities[267].

Secondly, TNF-α is able to increase adipocyte lipolysis, the process by which fats are broken down into FFAs[268,269]. FFAs promote the synthesis of fatty acid metabolites such

as diacylglycerol, ceramides, and fatty acyl-CoAs[109,270]. These fatty acid metabolites can in turn activate several serine/threonine kinases, including PKC, and NF-κB[109,270]. These kinases have the ability to phosphorylate proteins in the insulin signalling pathway, such as the IRS-1, GSK3, and Akt (refer to Section 1.2)[109,270]. PKCs are responsible for the phosphorylation of IRS-1 at some of its serine residues either directly or indirectly, including Ser24, Ser270, Ser318, Ser612, Ser632, Ser789, and Ser1101, which prevents threonine phosphorylation resulting in the inactivation of IRS-1[17,58,17]. Consequently, this leads to further inhibition of downstream signalling affecting PI3K. Studies have shown that FFAs can activate various PKCs that induce IRS-1 serine phosphorylation and as a result, impair insulin signalling[271–274]. This finding could explain the reduced cellular glucose uptake demonstrated in humans, as an unperturbed PI3K/Akt pathway is responsible for the translocation of glucose transporters to the cell membrane facilitating the import of glucose into the cell[58,275,276]. Research has also shown that FFAs can lead to the phosphorylation of Akt at Thr34, preventing phosphorylation at its Thr308 and Ser473 sites, which is required for its activation leading to cells becoming unresponsive to insulin[277,278].

Finally, Jun amino-terminal kinases (JNKs) can be activated by TNF-α (Figure 1.6)[279–281]. JNK can directly phosphorylate IRS-1 at Ser307, and in doing so, reduce tyrosine phosphorylation, similar to what has been described above for PKC and IKK. This results in the inability of IRS-1 to interact with the IR in response to insulin, attenuating any further downstream signalling [282,283]. Furthermore, TNF-α-activated JNK acts along a positive feedback mechanism, whereby JNK stimulates NF-κB action, the transcription factor involved in the transcription of TNF-α and IL-6[282]. Studies have shown that activation of the JNK pathway decreases insulin sensitivity in mice[284,285]. In addition, it has been found that JNK activity is abnormally upregulated in obesity[279,284].
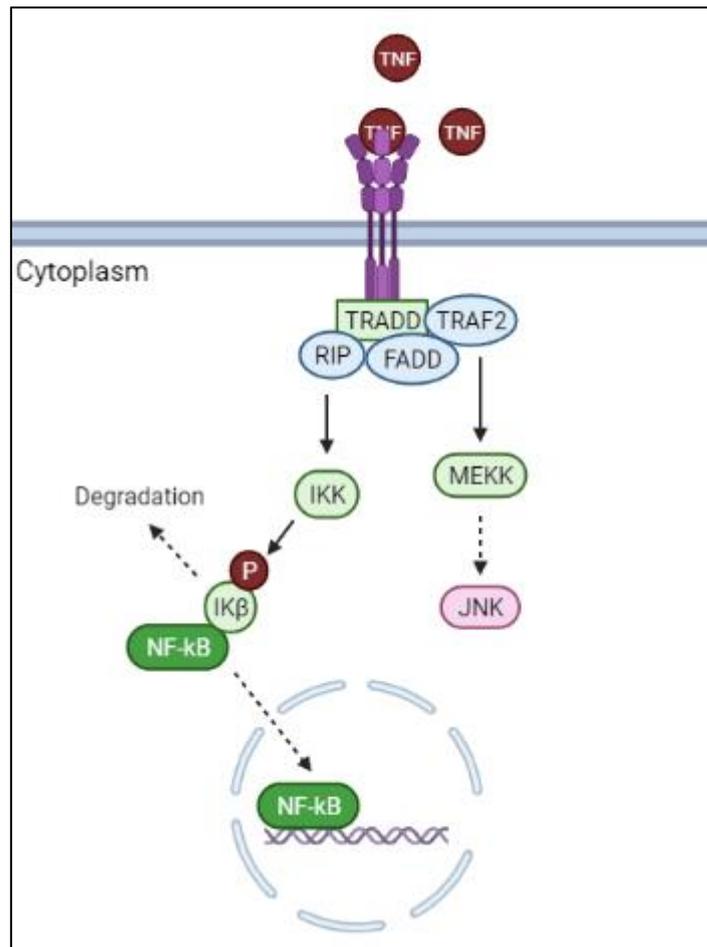
Figure 1.6. Schematic illustration of TNF-α signalling and the activation of the IKK and JNK pathways. TNF-α activates its receptor, TNFR-1, resulting in the recruitment of a scaffold protein TRADD. Activated TRADD recruits other proteins to the complex, namely, TRAF2, FADD, and RIP. TRAF2 summons IKK to the protein complex, whereby RIP is able to activate it. IKK subsequently phosphorylates IKβ, resulting in its dissociation from cytoplasmic NF-κB. NF-κB is free to enter the nucleus where it transcribes several genes. In addition, TRAF2 is responsible for activation of the MEKK pathway, resulting in downstream activation of JNK, a protein kinase. Image adapted from Wu, Y. and Zhou, B.P.[286], and Palladino, M. A. *et al.*[287]. Image drawn in Biorender.

1.4.5.2 IL-6 in insulin signalling

The pro-inflammatory cytokine IL-6 is involved in the immune response, such as B and T cell differentiation, the production of APPs, such as serum amyloid A (SAA) and CRP, and hematopoiesis[269,288]. Of the pro-inflammatory cytokines, IL-6 has shown to have the strongest correlation to insulin resistance, adiposity, and T2DM[289,290].

IL-6 binds to its IL-6 receptor (IL-6R) which allows the receptor to form a complex with another transmembrane protein known as glycoprotein 130 (gp130) as seen in Figure 1.7[290,291]. Upon complex formation, Janus kinase (JAK) proteins associated with gp130 become active, resulting in the phosphorylation of gp130 at tyrosine residues[290,292]. The phosphorylated tyrosine residues of gp130 become docking sites for molecules with

33

SH2 domains, such as Signal Transducer and Activator of Transcription 1 (STAT1) and STAT3[290–292]. The STAT transcription factors induce the expression of Suppressor of Cytokine Signalling (SOCS) proteins, which act *via* a negative feedback system to suppress the signal of cytokines[290,293]. SOCS also can negatively regulate insulin signalling. Studies have found that overexpression of SOCS results in a decrease in phosphorylation of tyrosine residues of IRS-1, leading to the inhibition of PI3K signalling activity[293–295]. Furthermore, inhibition of SOCS leads to improved insulin sensitivity in mice[296,297]. The mechanism whereby SOCS inhibits insulin signalling has been shown to involve the binding of SOCS to the IR thereby preventing the phosphorylation of IRS-1[298,299].

To summarise, the link between chronic inflammation and insulin resistance is evident albeit poorly understood. The increase in pro-inflammatory mediators such as TNF-α and IL-6 that persist in chronic inflammatory states do however provide some evidence in how chronic low-grade inflammation could result in insulin resistance. Further research into the effects pro-inflammatory cytokines have on other key proteins in the insulin signalling pathway are therefore of growing interest.
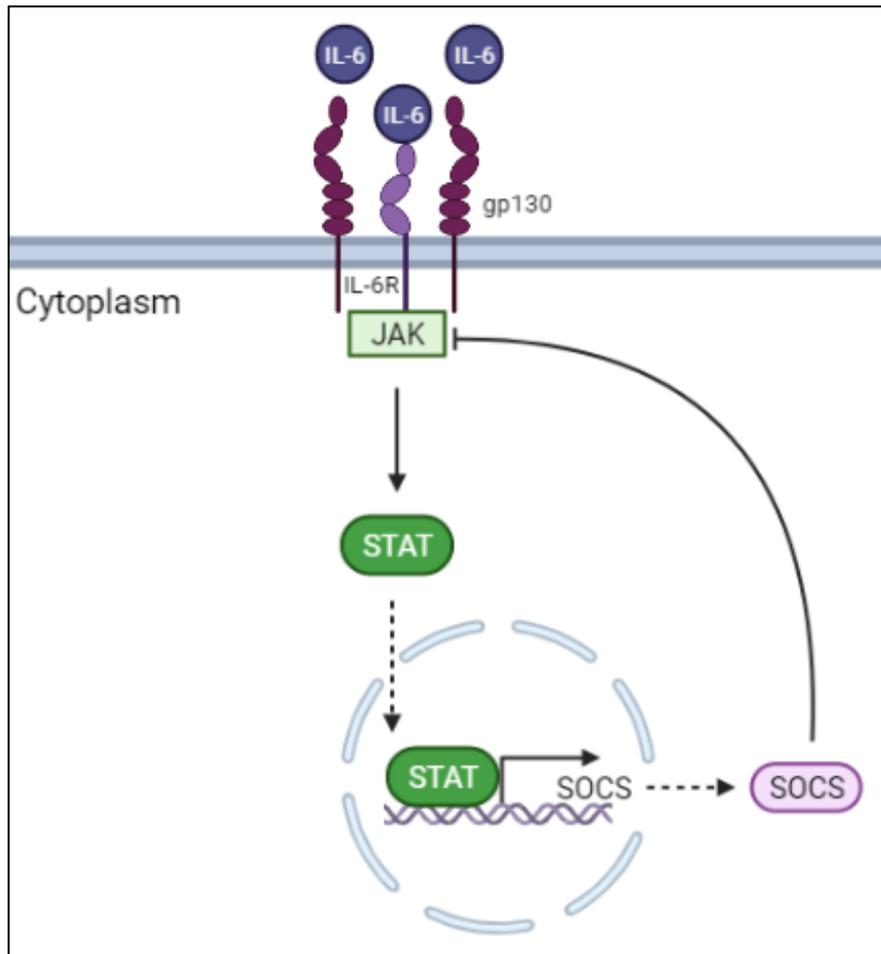
Figure 1.7. IL-6 signalling and the subsequent activation of the JAK/STAT pathway. IL-6 binds to its receptor, IL-6R, allowing the receptor to form a complex with the transmembrane protein, gp130. The complex recruits JAK proteins and upon associating with gp130, phosphorylate it as its tyrosine residues. These phosphorylated sites allow STAT transcription factors to dock to gp130, resulting in activation. STATs can subsequently enter the nucleus and initiate the transcription of various proteins, including SOCS.

1.5 The HPA axis and the stress response

1.5.1 The HPA axis in response to stress As mentioned previously, homeostasis is referred to as any self-regulating process by which biological systems tend to maintain stability while adjusting to conditions that are optimal for survival[27]. Homeostasis is continuously challenged by both intrinsic and extrinsic factors, termed stressors[300,301]. Stressors can be physical or emotional, resulting in activation of the stress response[300,301]. A key component in the stress response is the HPA axis (Figure 1.8)[302]. Stressors are responsible for the stimulation of neurons present in the paraventricular nucleus (PVN) of the hypothalamus – this results in the secretion of two hormones namely, corticotropin-releasing hormone (CRH), and arginine vasopressin

(AVP)[300,302,303]. Both AVP and CRH act on their specific receptors on the anterior pituitary gland, stimulating the secretion of adrenocorticotropic hormone (ACTH)[302,304,305]. The main target of ACTH is the adrenal cortex, where it induces the synthesis and secretion of the GC, F[302–304]. GCs play a key regulatory role on the activity of the HPA axis and the termination of the stress response *via* a negative feedback loop at the hypothalamus and pituitary[300,303]. This ultimately signals for the HPA axis activity to return to normal. The action of GCs such as F is of particular interest in the stress response.
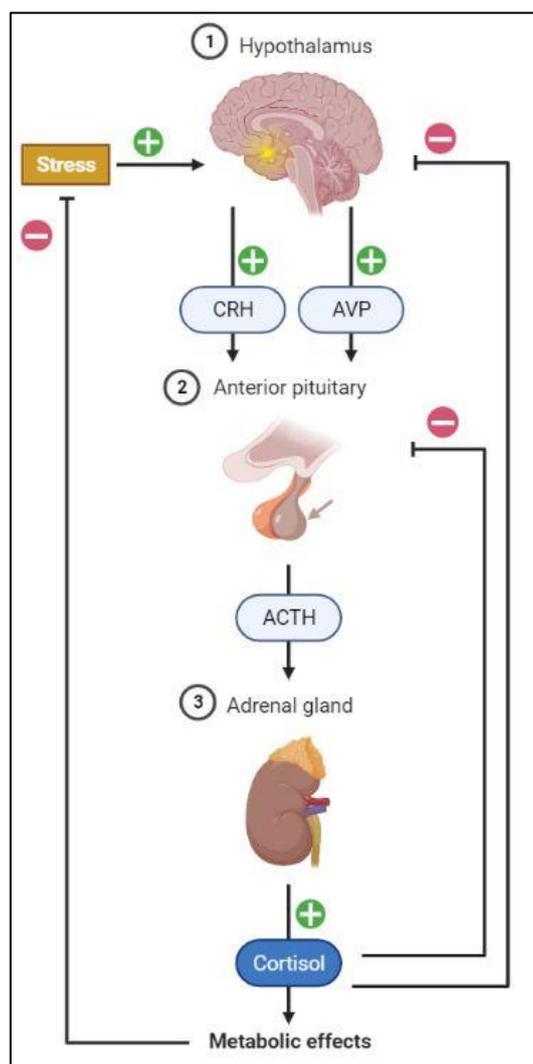
Figure 1.8. Schematic illustration of the HPA axis and the regulation of the stress response. Stressors stimulate neurons on the PVN of the hypothalamus resulting in the secretion of CRH and AVP. Both CRH and AVP bind to their cognate receptors at the anterior pituitary, causing secretion of ACTH. ACTH travels to the adrenal gland where it stimulates the release of F. F regulates several metabolic processes. F operates on a negative feedback loop, where it inhibits further secretion of CRH and AVP at the hypothalamus, and ACTH at the pituitary, thereby attenuating further secretion of F and ending the stress response. Image adapted from Tsigos and Chrousos[300]. Image drawn in Biorender.

1.5.2 Glucocorticoids in the stress response

GCs are cholesterol-derived hormones that play a critical role in maintaining stress-related homeostasis[306]. These molecules play a role in several other functions including growth, metabolism, cognition, and immunity[306,307]. GCs are able to exert their influence through the glucocorticoid receptor (GR), a transcription factor belonging to the nuclear receptor family[306,307]. The GR is found inactive in the cytoplasm as part of a multi-protein complex. Upon ligand binding, cytoplasmic GR becomes active, dissociates from its multi-protein complex, and translocates to the nucleus[306–308]. Once the GR enters the nucleus, active GR can associate with target genes by either direct binding to DNA *via* GREs, or through protein-protein interaction with transcription factors (Figure 1.9)[306–308]. Binding to GREs result in the activation of transcription, often termed 'transactivation', or the inhibition of transcription ('transrepression'). A good example of transrepression *via* GR is the negative feedback regulatory mechanism of the HPA axis that represses the production of ACTH. The F-activated GR binds directly to a negative GRE site of the proopiomelanocortin gene, inhibiting its transcription[308,309]. Proopiomelanocortin is the precursor for many peptides and hormones, including ACTH[310]. While it is largely unknown how F inhibits CRH *via* the GR on a genomic level, there is evidence suggesting F inhibits CRH synthesis and secretion from the PVN *via* membrane-bound GR[311]. GC action that results in the transactivation of genes is seen in numerous metabolic processes. For example, upon GC activation, the GR can induce the transcription of the PEPCK gene, resulting in an increase in gluconeogenesis[312,313]. The role GCs play in the regulation of metabolism is of importance to this thesis, especially stress-induced changes to glucose metabolism.
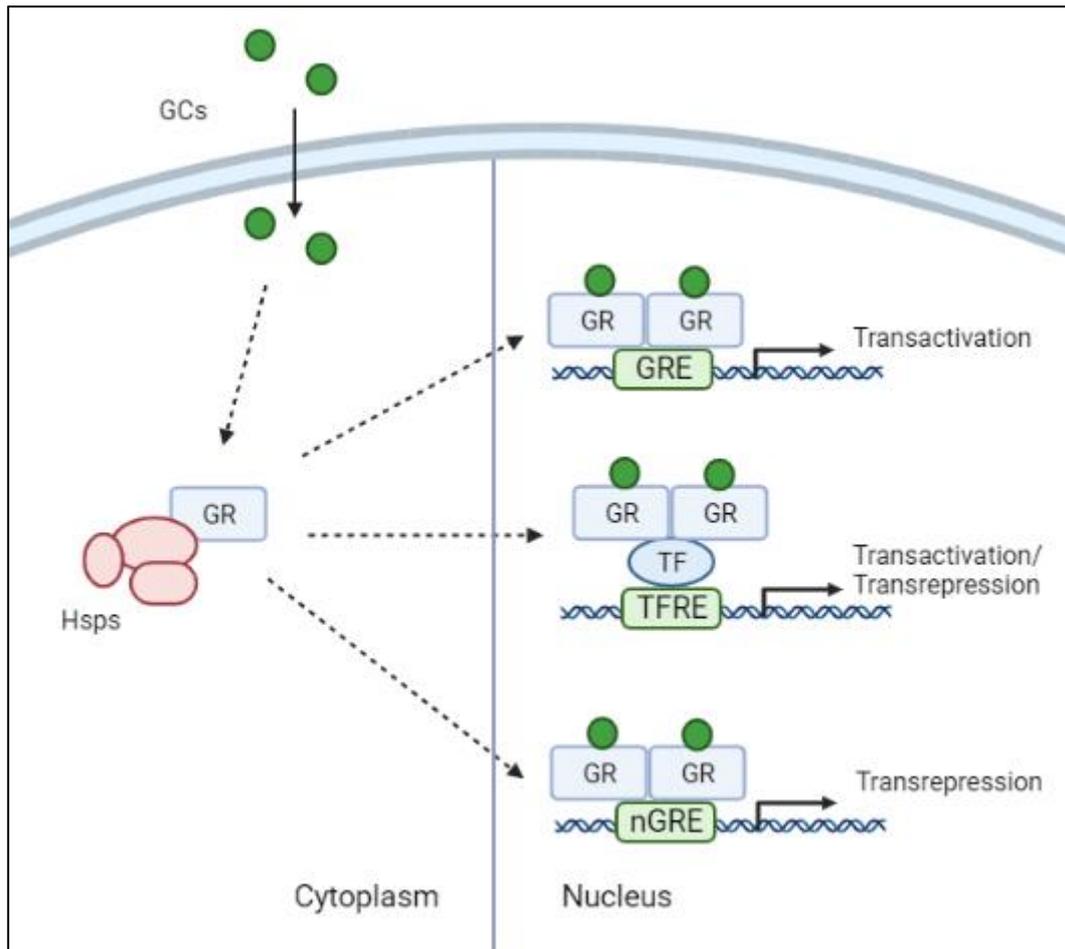
Figure 1.9. Schematic illustration of GC-activated GR and the regulation of transcription. The presence of GCs results in the activation of the GR in the cytoplasm by causing dissociation of the multi-protein complex. Activated GR enters the nucleus where it can either bind directly to GREs, bind to a transcription factor, thereby eliciting a response *via* other Transcription Factor Response Element (TFRE), and GR can bind to a negative GRE (nGRE). Image adapted from Dinarello *et al.*[314] Image drawn in Biorender.

1.5.3 How stress affects metabolism

As mentioned previously, a stressor, whether physiological or psychological, results in a signalling cascade that releases GCs from the adrenal gland[315]. During perceived stress, tissues increase their metabolic needs, thereby increasing the energy demands of the body. GCs act to provide this elevated energy demand through increasing the production of substrates such as glucose, amino acids, glycerol, and FAs in various tissue types as summarised in Figure 1.10[315]. GCs increase lipolysis by activating the transcription of the adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) genes in adipocytes. Both ATGL and HSL are involved in the breakdown of triacylglycerol into FAs and glycerol[315–317]. GCs also increase proteolysis through regulation of the ubiquitin-proteasome pathway[318–320]. In addition, GCs increase

proteolysis *via* the Ca^{2+}-dependent proteolytic pathway[318,319]. Furthermore, GCs can upregulate the expression of FOXO1 mRNA and protein[174,318,320–322].

GCs are especially well-known for their ability to regulate glucose homeostasis. GCs promote gluconeogenesis in the liver by increasing the expression of various gluconeogenic genes including PEPCK, G6Pase, and FBPase *via* the ligand-activated GR[316,323,324]. As a result, blood glucose increases to provide the body with enough energy[302,325]. It should be noted that FOXO1 plays a key role in transcribing the genes of PEPCK and G6Pase enzymes as mentioned earlier[326,327]. Furthermore, GCs inhibit glucose uptake in both skeletal muscle and adipose tissue by preventing the translocation of the glucose transporter, GLUT4[316,323,328]. In addition to this, GCs also function to inhibit the secretion of insulin from β-cells of the pancreas[316,323,329]. Taken together, stress-induced GC secretion plays an instrumental role in shifting the homeostasis of several metabolic functions. The role chronic stress plays on insulin signalling is of particular interest and will be elaborated on below.
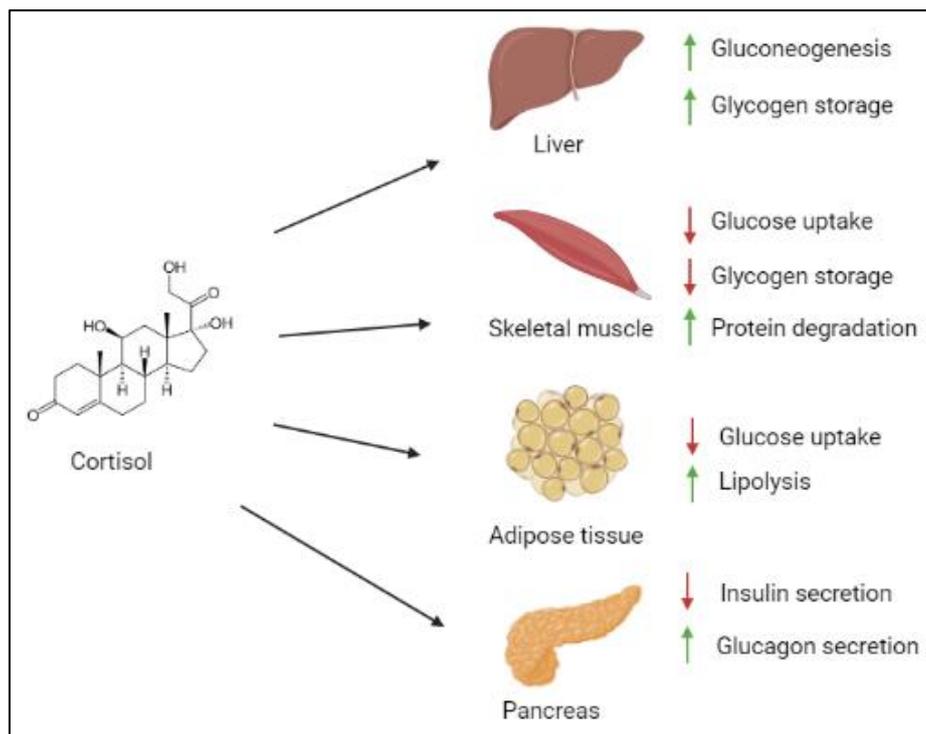
Figure 1.10. The effects GCs have on various tissue types to meet energy demands in response to stress. The endogenous GC, F, promotes gluconeogenesis in the liver by increasing the expression of PEPCK and G6Pase, as well as increases the rate of glycogenesis. In skeletal muscle, F reduces glucose uptake and the storage of glycogen, but increases protein degradation to supply energy. F also reduces glucose uptake in adipocytes but promotes lipolysis to provide fats for energy. F reduces the secretion of insulin and promotes glucagon secretion in the pancreas to regulate glucose metabolism. Image adapted from Kuo, *et al*[323]. Image drawn in Biorender.

1.5.4 Stress and inflammation

It is evident that GCs alter various metabolic functions in response to stress, therefore it is important to investigate what the body perceives as stress. Especially interesting is the relationship between the stress response and inflammation (Figure 1.11). As mentioned in the previous section, the immune response is tightly regulated *via* cytokine signalling. Pro-inflammatory cytokines are not only involved in the control of the immune response but serve as messengers from the immune system to the neuroendocrine system[330–332]. These inflammatory mediators *via* their cognate receptors act on the HPA axis resulting in the secretion of GCs[331,333]. GCs in turn act to increase the expression of anti-inflammatory cytokines and inhibit the secretion of pro-inflammatory cytokines[331]. For example, the activation of the GR *via* GCs leads to the transrepression of both TNF-α and IL-6 gene transcription. The GR interacts with NF-κB, the transcription factor involved in the production of both TNF-α and IL-6, through the process of tethering thereby preventing the transcription of these cytokines, which leads to an abated pro-inflammatory response[331,334,335]. Furthermore, GCs can mediate the increase in expression of anti-inflammatory mediators such as IκB, IL-4, and IL-10[336–339]. Unsurprisingly, GCs are commonly used as drug treatments in many inflammatory diseases such as rheumatoid arthritis and asthma[340–342;316,343,344]. Together, cytokines and GCs act to fight off the infection and allow the inflammatory response to subside once it is no longer needed, allowing the body to return to homeostasis. Conditions that shift homeostasis so that the stress response remains active however, can have serious consequences.
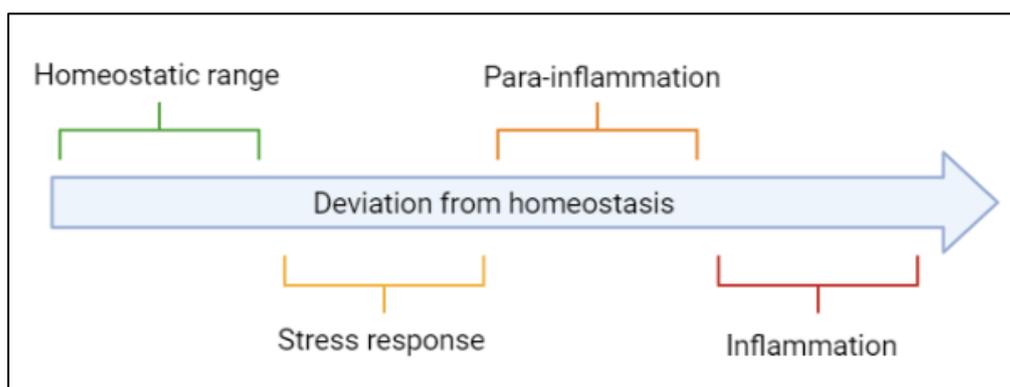
Figure 1.11. The progression of inflammation from homeostasis. The stress response is triggered when a stressor perturbs homeostasis beyond the norm. In addition to this, inflammatory mediators are active at the site of infection in an attempt to eliminate the stressor. Inflammation pursues to further promote the immune response. If inflammation persists for long periods of time, the homeostatic range shifts and results in a pathological state. Image adapted from Chovatiya & Medzhitov[221]. Image drawn in Biorender.

1.5.5 Chronic stress and insulin resistance

Chronic stress is loosely defined as prolonged or recurrent exposure to real or perceived threats that causes shifts in homeostasis and/or an individual's well-being[325]. This leads to extended hormone secretion, including F, by the HPA axis[302,325,345]. Chronic stress and the excess production of F has been linked to many pathological conditions, including Cushing's syndrome, depression, hyperthyroidism, or T2DM[302]. The persistent presence of inflammation has also been implicated in the chronic stress response, and *vice versa*[6,302,346]. A prime example is obesity. It is well-known that adipocytes secrete cytokines that are categorized as pro-inflammatory, such as TNF-α and IL-6[6,347,348]. An increase in visceral fat will result in an increase in the secretion of these cytokines, ultimately resulting in a chronic, low-grade inflammatory state in obese individuals[347–349]. The secretion of these pro-inflammatory cytokines triggers the release of GCs. As mentioned previously, GCs *via* the GR increase the promoter activity of genes involved in glucose metabolism. The increase in glucose is to provide the body with enough energy during the stress event[302,325]. Due to the chronic inflammatory state however, F levels remain elevated and result in elevated blood glucose, beyond the homeostatic threshold, commonly referred to as hyperglycemia[323,350,351]. For example, Cushing's Syndrome, a disease characterized by hypercortisolism, is very commonly associated with hyperglycaemia[352–354]. Furthermore, several studies have found that chronic stress can lead to low-grade inflammation[355–358]. While the precise mechanism is unclear, it is strongly thought that cells become less sensitive to GCs and as a result become resistant to its effects, and that the increase in pro-inflammatory cytokines is no longer hindered by further production of GCs in a negative feedback manner. This results in the inability of GCs to terminate the inflammatory response in immune cells[10,346,359,360]. Hyperglycaemia and insulin resistance are closely linked, as abnormally elevated blood glucose affects the insulin sensitivity of cells[77,361,362]. Furthermore, chronic stress has been implicated in insulin resistance. Several case-studies have found that individuals who suffer from psychological stressors have an increased association with insulin resistance[363–367].

In closing, although GCs are known to antagonise pro-inflammatory cytokine signalling, both stress and inflammation have been implicated in the development of insulin resistance, and it is evident that chronic stress and low-grade inflammation cannot be considered isolated in metabolic dysregulation. Hepatic insulin resistance

especially has been linked to the dysregulation of FOXO1, a transcription factor responsible for expression of key gluconeogenic enzymes[60,138–141]. Interestingly, GCs associated with the stress response are known to increase FOXO1 expression[318,320–322].The ability of GCs to regulate FOXO1 at the transcriptional level, could also contribute to the insulin resistant phenotype associated with GCs. Unfortunately, to the best of our knowledge no studies have linked an increase in FOXO1 expression in response to GCs to cells becoming insulin resistant. The effects of stress and inflammation on FOXO1 will be discussed in more detail next.

1.6 Effects of stress and inflammation on FOXO1

1.6.1 The effects of stress on FOXO1

As mentioned previously (Section 1.5), the endogenous human GC, F is involved in several important functions including metabolism. Elevated F levels over long periods of time i.e., chronic stress, has been known to increase the risk for the development of insulin resistance[367–370]. It is well established that GCs *via* the GR activate gluconeogenesis by directly upregulating the transcription of gluconeogenic genes (*g6pc* and *pck1*)[316,323,324]. Indirect transcriptional activation of these gluconeogenic enzymes also occurs as GCs also increase FOXO1 mRNA and protein levels, which as mentioned earlier is also responsible for the increase in G6Pase and PEPCK expression[174,318,320–322,371]. Cattaneo *et al.*, showed that pre-treatment with F resulted in a significant 4-fold increase in FOXO1 mRNA levels[371]. The synthetic GC, Dex, like F has also been shown to increase FOXO1 mRNA expression as well as its protein levels[174,321,322]. Furthermore, Liu *et al.* showed that mice subjected to stress for 6 weeks had elevated FOXO1 protein and mRNA levels[372]. The manner in which GCs regulate FOXO1 expression is direct as the *foxo1* gene promoter contains multiple GREs to which the ligand-activated GR can bind thereby regulating its transcription[174].

In addition to increasing FOXO1 levels through transcription, GCs have also been shown to affect FOXO1 post-translationally. Decreased phosphorylated FOXO1 levels have been observed in the liver of stress-induced mice indicative of active FOXO1[372]. In summary, there is sufficient evidence to suggest that stress, acting through GCs, positively influence the FOXO1 transcription factor at both the transcriptional level as well as post-translationally. This influence is of great interest, as it suggests that GC-

induced insulin resistance might be in part due to increased FOXO1 levels and activation.

1.6.2 The effects of inflammation on FOXO1

Chronic inflammation and the persistent secretion of cytokines such as IL-6 and TNF-α play a role in altering metabolic functioning. As mentioned previously, chronic inflammation often leads to the development of insulin resistance. The continuous secretion of cytokines activates a number of pathways (i.e., NF-κB and JNK) that in turn influence multiple nodes in the insulin signalling pathway. Furthermore, cytokines can also directly influence the Akt signalling pathway at several sites. Whether these cytokines influence FOXO1 levels, either directly *via* increased FOXO1 synthesis, or indirectly by preventing phosphorylation and subsequent ubiquitination followed by proteasomal degradation of FOXO1, could add to the multiple molecular mechanisms whereby pro-inflammatory cytokines can induce insulin resistance.

Very few studies have investigated the effects of pro-inflammatory cytokines on FOXO1 expression and activity, with the majority of these focussing on the effect of TNF-α on FOXO1. To the best of our knowledge, the role of IL-6 on FOXO1 activation and mRNA or protein levels have not been investigated.

In regard to TNF-α, contrasting effects on FOXO1 mRNA expression have been reported. A study by Alikhani *et al.,* showed that TNF-α increased FOXO1 mRNA expression and its activity in human fibroblasts[373], whilst another study by Miao *et al*., observed no effect at the mRNA level or protein level in human liver cells. The latter study did however observe that TNF-α treatment lead to the activation of FOXO1 by interfering with insulin/Akt signalling and consequently inhibiting phosphorylation of FOXO1. Additionally, these authors observed no *foxo1* promoter activity[374]. These differences could be attributed to tissue specific effects. Nonetheless, several studies demonstrated an increase in TNF-α induced FOXO1 activity, linking inflammation to FOXO1 activity[375–377]. In addition, some investigations have demonstrated that TNF-α induced activation of FOXO1 leads to an increase in production of other pro-inflammatory cytokines such as IL-6, with increased FOXO1 expression in diabetes reported to potentiate pro-inflammatory cytokine production in insulin resistance[326,378]. Miao *et al*., proposed that FOXO1 leads to an increase in pro-inflammatory cytokines levels rather than cytokines increasing the production of FOXO1[374]. This is supported

by other studies that show FOXO1 increases expression of TNF-α, IL-6, and IL-1β[378–380]. This is in spite of evidence by others showing increase mRNA expression in response to TNF-α[376,381]. Thus, while it is clear that the FOXO1 transcription factor is linked to inflammation and the production of pro-inflammatory cytokines, literature is conflicting on whether pro-inflammatory cytokines such as TNF-α and IL-6 affect the expression of the FOXO1 transcription factor.

1.7 Conclusion

Chronic inflammation is commonly associated with the development of insulin resistance and T2DM[217,218,382,383]. In addition, another factor that is known to contribute to insulin resistance is stress[367–369]. Interestingly, inflammation and the stress response are closely linked in maintaining homeostasis[7,221,384]. It is therefore odd that stress and inflammation is rarely looked at together in the development of insulin resistance. Insulin resistance is characterized by dysregulated insulin signalling, of which the FOXO1 transcription factor is part of[47,92,385]. In insulin resistance, FOXO1 continues to transcribe the genes of key enzymes required for gluconeogenesis and further contributing to hyperglycaemia[60,138,139]. Some studies show that GCs related to the stress response upregulate FOXO1 expression[174,318,320–322,371], but no research has investigated FOXO1 expression in stress-induced insulin resistant conditions. To add to this, FOXO1 is known to regulate gene expression of several pro-inflammatory cytokines[378–380], but research on FOXO1 expression induced by cytokines in inflammation-induced insulin resistance has yet to be elucidated. Understanding whether stress and inflammation affect FOXO1 regulation in insulin resistance and the mechanisms thereof could provide valuable insight towards the development of novel drug targets.

1.8 Hypotheses and aims

GCs and pro-inflammatory cytokines have shown to individually inhibit insulin signalling in various cell types including the liver[316,323,386,387]. However, how concomitant exposure to GCs and pro-inflammatory cytokines affect insulin signalling remains unclear and surprisingly overlooked. This is especially important considering that in a biological system both types of signalling molecules, albeit at different ratios, are always present in circulation. Furthermore, how GCs and pro-inflammatory cytokines affect downstream effects of insulin signalling such as the deactivation of FOXO1 are

not clear. Whether an insulin resistant state established by these signalling molecules influence FOXO1, a transcription factor responsible for the upregulation of key gluconeogenic enzymes, remains to be elucidated.

The literature clearly indicates that stress and inflammation are associated with the development of insulin resistance. Furthermore, insulin resistance is characterized by a defective insulin signalling pathway. In the liver, defective insulin signalling results in the inability to halt processes such as gluconeogenesis. Thus, the main aim of this study was to investigate the effects of stress and inflammation on the FOXO1 transcription factor, as it plays a key role in gluconeogenesis.

The current study hypothesizes that stress and inflammation, in the form of GCs and pro-inflammatory cytokines, can co-regulate FOXO1 transcription factor and as a result contribute to the increased risk of insulin resistance.

To prove or disprove this hypothesis, the following research questions were posed:

1   Are pro-inflammatory cytokines or GCs able to affect the insulin-induced activation of the key protein Akt to what is seen in insulin resistant states?

2   How is FOXO1 protein and mRNA expression affected in the presence of pro-inflammatory cytokines (TNF-α and IL-6) and/or GCs (Dex and F)?

3   Do GCs and/or pro-inflammatory cytokines influence the FOXO1 transcription factor at a transcriptional level?

4   Will the presence of pro-inflammatory cytokines and/or GCs alter the insulin-induced phosphorylation status of FOXO1?

The following aims have been established to address the above questions using the murine hepatoma cell line, BWTG3, and the human liver carcinoma cell line, HepG2, to investigate insulin signalling in hepatocytes:

Aim 1: Determine whether stress using the GCs, Dex, and F/Cort, or inflammation using the pro-inflammatory cytokines, TNF-α and IL-6, affect the ability of insulin to induce Akt phosphorylation.

Aim 2: Investigate whether FOXO1 mRNA and protein levels are affected by GCs in the absence and presence of the pro-inflammatory cytokines, TNF-α or IL-6.

Aim 3: Determine if FOXO1 is influenced by stress and inflammation at a transcriptional level, by investigating FOXO1 promoter activity in response to GCs and pro-inflammatory cytokine treatment.

Aim 4: Establish whether stress and inflammation affect insulin-induced phosphorylation of FOXO1.

The materials and methods that were used for this study are described in Chapter 2. The results for this study are shown in Chapter 3, followed by the discussion of these results in Chapter 4. In addition, the limitations and future research will be described in Chapter 4.

Chapter 2: Materials and Methods

2.1 Test compounds

The test compounds used included a synthetic GC, Dex ((11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione), and an endogenous GC, F (11β,17α,21-trihydroxypregn-4-ene-3,20-dione-17-hydroxycorticosterone), which were purchased from Sigma Aldrich. These stock solutions were prepared in ethanol to a final concentration of 1μM and stored at -20°C. Additionally, endogenous pro-inflammatory cytokines TNF-α and IL-6 were purchased from Thermo Fisher Scientific. Stock solutions of TNF-α were prepared in dH_2O to a final concentration of 1mg/ml and stored at -20°C. Stock solutions of IL-6 were prepared in acetic acid to a final concentration of 1mg/ml and stored at -20°C. Working solutions of 20ng/ml for both TNF-α and IL-6 were prepared using low glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS and stored at -20°C.

2.2 Plasmids

This study made use of a *foxo1* promoter reporter construct containing the wild-type mouse *foxo1* promoter in a pGL3 vector, named pGL3-mFOXO1wt-luc. This plasmid was kindly gifted by JB Demoulin at De Duve Institute, Université Catholique de Louvain (Brussels, Belgium).

2.3 Cell culture

Cell lines that were used in this study included a human hepatoma derived cell line (HepG2) and a mouse hepatoma derived cell line (BWTG3). The BWTG3 cells were gifts from the Laboratory for Eukaryotic Gene Expression and Signal Transduction, University of Ghent (Belgium), whilst the HepG2 cells were purchased from Cellonex.

2.3.1 Maintenance of cells

The HepG2 cell line was maintained in low glucose (5mM) DMEM containing sodium pyruvate (1mM) and sodium bicarbonate (17.9mM) (Sigma-Aldrich). The culture medium was also supplemented with 10% fetal bovine serum (FBS) (Merck) and 1% (v/v) penicillin/streptomycin (PenStrep) (50 IU/ml penicillin and 50 μg/ml streptomycin) (Sigma-Aldrich). The cell line was maintained at a temperature of 37°C, 95% humidity and 5% CO_2 in 75 cm^2 tissue culture flasks (SPL Life Sciences). All cell lines were

tested for mycoplasma using Hoechst Staining and found to be negative (see Addendum A).

2.3.2 Treatment conditions

For time course experiments, HepG2 cells were seeded into 12-well plates at a density of 4×10^5 cells/well and BWTG3 cells at a density of 2×10^5 cells/well and left to grow to a confluency of 70-80% in supplemented DMEM. This was followed by serum starving the cells for 24 hours in un-supplemented DMEM. Cells were treated with 100nM Dex, 100nM F, 20ng/ml TNF-α or 20ng/ml IL-6 for 2, 4, 24, 48, or 72 hours, followed by treatment with 100ng/ml insulin for 30 minutes prior to cell lysis to stimulate the insulin signalling pathway. Test compounds were added directly to each well containing un-supplemented DMEM. Due to the GC test compounds being made up in EtOH and the cytokine test compounds being made up in supplemented low-glucose DMEM, the vehicle control for each experiment consisted of the addition of supplemented low glucose DMEM to a final concentration of 0.1%, and EtOH. For the treatment conditions of the remainder of experiments, the cells were serum starved for 24 hours followed by treatment with GCs for 24 hours and cytokines for 4 hours before cell lysis. Insulin was added 30 minutes prior to cell lysis. All experiments were conducted on cells with a passage number between 1 - 29 for HepG2 and 1 – 35 for BWTG3.

2.3.3 Transfection of cell lines

HepG2 cells for promoter-reporter experiments were plated into 10cm^2 dishes at a density of 4×10^5 cells/well. After the transfection, HepG2 cells were re-plated into a 96-well plate at a density of 1×10^5 cells/well. Twenty-four hours after seeding and cells have reached 60-70% confluency, pGL3-mFOXO1wt-luc plasmid was transiently transfected using X-tremeGENE 9 transection reagent (1µg DNA to 2µl X-tremeGENE) as described by the manufacturer (Roche). During experiments, HepG2 cells were transfected in DMEM supplemented with 1% Pen/Strep and 10% FBS and left for 24 hours at 37°C, 95% humidity and 5% CO_2. After 24 hours, cells were replated into 96-well plates and left for an additional 24 hours. HepG2 cells were rinsed once with cold 1X phosphate buffered saline (PBS) before the prescribed treatments were added. Cells were washed with cold 1X PBS and harvested in passive lysis buffer (0.09M Tris pH 8.0, 0.5M EDTA, 10% glycerol, 0.2% (v/v) TritonX-100, 0.125M sodium fluoride (NaF), 50mM sodium orthovanadate (Na_3VO_4), 1% (v/v) phenylmethylsulphonyl fluoride (PMSF)).

2.4 Determining FOXO1 protein expression and analysing ligand-induced effects

Western blotting was used to determine cellular FOXO1 protein expression levels and P-FOXO1 protein expression levels in HepG2 cells in the absence and presence of test compounds. This technique was also used to determine whether the above-mentioned treatment conditions mimic an insulin-resistant state by looking at Akt phosphorylation status. The protein expression was normalized to a loading control to ensure equal protein loading between treatment conditions. Loading controls used were unaffected by the test compounds (Addendum C).

2.4.1 Preparation of lysates

After the prescribed seeding, transfections, and treatments, cells were washed with cold 1X PBS and harvested in passive lysis buffer. Protease inhibitors were added into the passive lysis buffer and additionally phosphatase inhibitors to samples that were to be probed for phosphorylation sites (Roche Applied Science). Once lysates were prepared and added to microcentrifuge tubes, samples underwent a freeze-thaw cycle, followed by adding 5X SDS-reducing lysis buffer (1M Tris-HCl pH 6.8, 10% SDS, 0.1% (w/v) bromophenol blue, 20% (v/v) glycerol). Lysates were boiled at 95°C for 10-15 minutes to denature proteins and then stored at -20°C for future use.

2.4.2 Western blotting

Protein lysates were thawed and then separated on 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) (30% acrylamide, 1.5M Tris-HCl pH 8.8, 1M Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED) using the BioRad MiniProtean gel system and power pack (BioRad). A constant voltage of 100V for 15 minutes, and 200V for 90 minutes was applied in a 1X running buffer (25mM Tris-HCl, 192mM glycine, 0.1% SDS). The Color Prestained Protein Standard Broad Range Ladder (Inqaba Biotec) was loaded to determine if the protein of interest was the expected size. After protein separation in the gels took place, the proteins were transferred to a Hybond-ECL nitrocellulose membrane (AEC Amersham) using the Hoefer™ semi-dry transfer system (Thermo Fisher Scientific) in cold 1X transfer buffer (25mM Tris, 192mM glycine, and 20% (v/v) methanol). After the transfer, membranes were blocked in 5% (w/v) fat-free milk powder or 5% bovine serum albumin (BSA) (Merck) prepared in 1X Tris Buffered Saline (TBS) (0.05M Tris-HCl, 0.15M NaCl) containing 0.1% (v/v)

Tween-20 (1X TBST) for 90 minutes at room temperature. Membranes were washed once with 1X TBST before being incubated with primary antibodies overnight at 4°C (Table 2.1). Anti-phospho Akt (Thr308) and anti-phospho FOXO1 (Ser256) were used to probe for phosphorylated proteins. Anti-FOXO1 and anti-Akt was used to probe for total protein. Anti-EF-1α1 and anti-Hsp90 were used to probe for proteins that were used as loading controls for normalization to confirm equal loading of proteins. All primary antibodies were made up in 1X TBST and stored at 4°C. Membranes were removed from primary antibodies and then washed 2 x 5 minutes with 1X TBST and 1 x 5 minutes with 1X TBS and then incubated with the appropriate secondary antibodies for 1.5 – 2 hours at room temperature. Secondary antibodies (Table 2.1) were prepared in 5% (w/v) fat-free milk powder. Blots were again washed 2 x 5 minutes TBST and 1 x 5 minutes TBS before visualization. Blots were submerged in BioRad ECL western blotting substrate (BioRad) for approximately 3 to 5 minutes before bands could be visualized using the chemiluminescence setting on the iBright™ Imaging System (Invitrogen). The My Image Analysis® version 2.0 (Thermo-Scientific) software was used to be able to calculate the intensity of the band representing the protein of interest.

Table 2.1. Antibodies used throughout this study:

Target protein	Size (kDa)	Primary antibody (1°)	1° antibody dilution	Secondary antibody (2°)	2° antibody dilution
FOXO1	80kDa	sc-514610, Santa Cruz	1:100	Goat anti-mouse IgG-HRP #ab97046, Abcam	1:10 000
EF-1α1	50kDa	sc-21758, Santa Cruz	1:1000		
Phospho-FOXO1	82kDa	#9461, CST	1:500	Goat anti-rabbit IgG-HRP #ab6791, Abcam	1:5000
Phospho-Akt (Thr308)	60kDa	#13038, CST	1:700		
Total Akt	60kDa	#9272, CST	1:1000		
Hsp90	90kDa	sc-7947, Santa Cruz	1:1000		

2.5 Determining FOXO1 mRNA expression and analysing ligand-induced effects

Real-time PCR (qPCR) was used to determine whether the presence of the selected test compounds alter the mRNA levels of FOXO1. Furthermore, qPCR was used to determine whether the mRNA levels of FOXO1 are altered when cells are treated with CHX prior to the addition of the test compounds. mRNA expression levels were normalized to a house-keeping gene, 18S, or GAPDH, that is unaffected by the treatment conditions.

2.5.1 RNA isolation

After treating cells accordingly, the cells were harvested for RNA. Cells were lysed in 400µl TRIzol reagent (Thermo Fisher) and stored at -20°C overnight. Cells were thawed on ice and then transferred to labelled microcentrifuge tubes to which 80µl of chloroform (Sigma-Aldrich) was added. Samples were vortexed for 1 minute before centrifugation at 4°C for 15 minutes at 14 000 rpm. Approximately 130µl (or as much as possible) of the clear supernatant was taken up and added to chilled RNase-free microcentrifuge tubes. Following this, 200µl of isopropanol (Merck) was added to each sample and then vortexed for 1 minute. Lysates were frozen at -20°C for 2-5 days to facilitate RNA precipitation. Samples were taken out of the freezer and placed in the centrifuge at 4°C for 30 minutes at 14 000 rpm to form an RNA pellet. The supernatant was removed and discarded. The remaining pellet was washed with 500µl 70% ethanol-Diethyl pyrocarbonate (DEPC) water and centrifuged at 4°C for 5 minutes at 14 000 rpm at least once, removing the supernatant each time. This ensures the removal of any contaminants. The pellet is centrifuged one last time so that any remaining liquid could be removed before the lysates were left to air-dry for 10-30 minutes. Samples were dissolved in 20µl of DEPC water and then placed on a heating block set to 55°C for 5 minutes. The RNA concentrations and quality (260/280 ratio) were measured using the NanoDrop ND-1000 (Thermo Fisher). The integrity of the isolated RNA was determined by loading 1µg RNA on a 1% (w/v) agarose gel containing Nancy-520® (Sigma Aldrich) in 1X Tris-acetate-Ethylenediaminetetraacetic acid (EDTA) (TAE) buffer diluted from a 50X TAE stock (Tris-HCl, glacial acetic acid, 0.5M EDTA pH 8.0). The agarose gel was visualized using the UV setting on the MyECL imager to confirm the presence of intact 28S and 18S ribosomal RNA (Addendum A).

2.5.2 cDNA synthesis

cDNA was synthesized from the 1µg of RNA isolated previously using the ImProm-II reverse transcription system (Promega) according to the manufacturer's protocol. The RNA was added to a PCR tube using a calculated volume constituting 1µg. oligoDT primers (50µg/ml) were added, followed by the addition of nuclease-free water to make up a final volume of 5µl. The contents were incubated for 5 minutes at 70°C. After this, a master mix was added to each of the samples for a final volume of 20µl. The master mix contained: 7.6µl DEPC-water, 4µl ImProm-II 5X reaction buffer, 1.5 mM $MgCl_2$, 0.67 mM dNTPs, 20U recombinant RNasin® ribonuclease inhibitor and 160U ImProm-II reverse transcriptase. To allow the oligoDT primers to anneal, the mixture was placed on a heating block at 25°C for 5 minutes. Following this, the samples were placed at 45°C for 1 hour to allow for extension. Finally, the mixture was placed at 70°C for 15 minutes and then on ice for 5 minutes to inactivate the reverse transcriptase. cDNA samples were stored at -20°C for future use.

2.5.3 Quantitative Real-time PCR (qPCR)

Primers specific for FOXO1 (Table 2.2) were designed using National Center for Biotechnology Information (NCBI) and then purchased from Inqaba Biotec, or Integrated DNA Technologies (IDT). Primers were dissolved in 1X Tris-EDTA (TE) buffer to make a primer stock solution of 100µM from which the working solution of 10µM of each primer was made for cDNA synthesis. qPCR was performed to measure the expression of the FOXO1 gene from cDNA prepared. Each 10µl reaction consisted of 1µg cDNA, 0.5µl forward primer (10µM) and 0.5µl reverse primer (10µM), 1.75µl DEPC water and 6.25µl of SYBR green (Merck). The annealing temperatures for each primer set were optimized (Addendum A) and the primer efficiencies determined (Table 2.2) by preparing a standard curve from a dilution series of cDNA previously prepared. The expression of the FOXO1 gene was normalized to the 18S or GAPDH reference gene.

Table 2.2. Primers used throughout this study:

Species	Protein	5'-3' forward primer sequence	5'-3' reverse primer sequence	Optimized annealing temperature (°C)	Product size	Primer efficiency
Human	FOXO1	TTC ACC CAG CCC AAA CTA CC	ATA ACT GTG CGC CTG GAC TC	63	139	2.76
Human	18S	GAG AAA CGG CTA CCA CAT CCA AG	CCT CCA ATG GAT CCT CGT TA	60	158	2.05
Human	GAPDH	TGA ACG GGA AGC TCA CTG G	TCC ACC ACC CTG TTG CTG TA	58	307	2

2.6 Investigating ligand activity on the FOXO1 promoter

The promoter-reporter assay experiments were performed to determine whether the test compounds representing stress and inflammation influence the FOXO1 transcription factor at its promoter. Cells were transfected with a plasmid containing the mouse wild-type FOXO1 promoter (pGL3-mFOXO1wt-luc) prior to treatments with the test compounds and the resulting relative light units (RLUs) were normalized to a transfected but untreated vehicle control. The total protein in each well was determined using a bicinchoninic acid (BCA) assay and used to normalize the resulting RLUs.

2.6.1 Promoter-reporter assay

HepG2 cells were seeded into 10cm^2 dishes and transfected at 60 – 70% confluency as described above. After treatments in the 96-well plate, cells were rinsed once in cold 1X PBS and stored at -20°C overnight to promote cell lysis. After thawing the 96-well plates, 5µl of each sample was added to white 96-well plates. Once the plates were clipped into the Luminometer and the experiment started, 25µl PROMEGA luciferase assay substrate (Anatech) was injected into each well containing sample with an integration time of 8 seconds and a delay after measurement of 2 seconds, and RLUs were recorded. The RLUs of each treatment condition was normalized to the RLUs of the vehicle control.

2.6.2 BCA analysis

To normalize the RLU values determined by the Luminometer, the total protein content of each well was quantified using the Pierce™ BCA protein assay (Thermo Fisher Scientific). Bovine serum albumin (BSA) standards were initially prepared by making 8 serial dilutions from a 1ml BSA stock ranging in concentration from 2mg/ml to 0.25mg/ml. The diluent used was passive lysis buffer. Following this, the BCA working reagent was prepared by combining reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1M sodium hydroxide) and reagent B (4% cupric sulphate) in a 50:1 ratio. In a 96-well microplate, 10µl of cell lysate was added into the wells of the microplate along with 10µl of each serial dilution in triplicate. Following this, 200µl of the BCA working reagent was added to the samples in the microplate and incubated at 37°C for 30 minutes covered in foil due to the light sensitivity of the working reagent. The absorbance of each sample was measured at 562nm using the Multiskan SkyHigh microplate reader (Thermo Fisher). A standard curve was determined using the absorbance of the standards in GraphPad Prism® (version 8). The protein concentration of each treated cell lysate plated could be determined by interpolating the absorbance into the standard curve generated and using the equation of the linear region given (the Beer-Lambert law was taken into consideration). Thus, the RLUs of a given cell lysate were normalized to the total protein concentration of the cell lysate.

2.7 Statistical analysis

Statistical analysis used throughout this study made use of GraphPad Prism software (Version 8). The statistical analysis applied to each experiment are given in detail in the figure legends. The statistical significance is indicated using numerical symbols (e.g., * or #), with the value they represent indicated in the figure legends. All data are presented as mean with error signifying standard deviation (SD). No statistical analysis was performed on data with only two independent experiments.

Chapter 3: Results

3.1 Introduction

The human hepatoma cell line, HepG2, are commonly used in insulin signalling studies due to their responses, particularly gluconeogenic and hepatokine gene expression, being closely related to what is described in *in vivo* studies[388,389]. For this reason, the HepG2 cell line was chosen to investigate FOXO1 expression and activity in an insulin resistant state, which for the purposes of this study was determined as the inability of insulin to activate Akt in response to the test compounds. The mouse hepatoma cell line, BWTG3, have to the best of our knowledge, not been used in previous research to study insulin signalling. Their use in insulin signalling have thus been optimized previously by our research group (unpublished data) [390].

A low glucose (5mM) culture medium was selected to maintain the hepatoma cells, as previous literature shows higher concentrations (25mM or higher) of glucose in culture medium can interfere with insulin signalling, including inhibiting the ability of insulin to stimulate the PI3K/Akt pathway[391,392]. This was also confirmed by our research group in the BWTG3 cells (unpublished data)[390]. As this study wanted to determine whether stress or inflammation could induce insulin resistance by inhibiting insulin-induced Akt phosphorylation, it was important to eliminate the influence of glucose as a variable.

To simulate the stress response, the GCs selected were Dex, F in the HepG2, and corticosterone (Cort) in the BWTG3. Dex, a synthetic analogue of endogenous GCs, is commonly used as a test compound due to its potency and long half-life[393,394]. Furthermore, several studies show that Dex can influence insulin signalling in liver cell culture[395–398]. Some studies even show that Dex can induce insulin resistance[399–401]. Thus, whether Dex could induce insulin resistance by inhibiting insulin-induced Akt phosphorylation in the HepG2 cells, as well as how these treatment conditions affect FOXO1 expression and activity was explored. Similarly, the influence of endogenous GC was also included in this study in addition to Dex. The endogenous GC, F, is elevated in insulin resistant patients[402–404]. Furthermore, F has been described to induce insulin resistance[351,405–407]. Cort is the endogenous GC in rodents and has also been reported to induce insulin resistance in rodents[408–410]. For this reason, the influence of endogenous GC-induced insulin resistance on FOXO1 was investigated, in addition to Dex.

In addition to chronic stress, chronic inflammation is also associated with the development of insulin resistance[382,411,412]. The pro-inflammatory cytokines TNF-α and IL-6 are elevated in insulin resistant states[217,411]. Moreover, some studies associate elevated TNF-α and IL-6 levels to contribute to an insulin resistant state[217,382,411]. Interestingly, no studies have investigated cytokine-induced insulin resistant effects on FOXO1. In addition, the influence of both stress and inflammation in combination, represented by the GCs and cytokines, on FOXO1 expression has not been investigated to the best of our knowledge. The combination treatments on FOXO1 in liver cells was further explored in this study.

3.2 Determining the effects stress and inflammation have on insulin-induced Akt activation

Optimal insulin signalling participates in the transcriptional regulation of various genes involved in glucose metabolism. The ability of insulin to regulate proteins in this pathway is what determines the responsiveness of a cell to insulin. The insulin signalling pathway is initiated upon insulin binding to the IR, resulting in a phosphorylation cascade. Within this cascade is Akt, and when phosphorylated, can regulate several sub-branches of the insulin signalling pathway including the transcriptional activity of FOXO1 (Figure 1.2). The phosphorylation status of Akt is often a key marker in determining the insulin responsiveness of cells[413–416]. Insulin resistance is frequently characterized as the inability of insulin to activate Akt *via* phosphorylation[417–421]. Thus, guided by the literature, for the purposes of this study the liver cells were considered insulin resistant when the ability of insulin to activate Akt in response to the test compounds was attenuated.

Both GCs and pro-inflammatory cytokines have been implicated in the development of insulin resistance. For this reason, the aim of this experiment was to establish an insulin resistant state (i.e., reduced insulin-induced Akt phosphorylation) in either one of the hepatoma cell lines, BWTG3 and HepG2, induced by stress or inflammation. These treatment conditions would then be used as an experimental model for all subsequent experiments.

In the current study, the stress response was represented by the GCs Dex, F, and Cort. F (human) and Cort (mouse) are endogenous GCs associated with the stress response and includes several other functions such as reducing inflammation. Dex is

a synthetic GC often used for its anti-inflammatory properties as it is intended to mimic the effects of endogenous GCs such as F and Cort[422–425]. The effect these GCs have on the phosphorylation status of Akt over time was investigated to establish a GC-induced insulin resistant state (i.e., reduced insulin-induced Akt phosphorylation). Additionally, total Akt protein levels in the HepG2 cells were also determined in response to GC treatment. Similar experiments were conducted to establish inflammation-induced insulin resistance. An inflammatory state was represented by treating the cells with the pro-inflammatory cytokines TNF-α and IL-6, as mentioned earlier. Both these inflammatory mediators are known to display elevated levels in insulin resistance[289,426–428].

The activation of Akt *via* phosphorylation and total protein levels were examined using western blot analysis. To analyse the effects of stress and inflammation on insulin-induced Akt regulation in HepG2 and BWTG3 cells, phosphorylation and total Akt levels in response to 100ng/ml insulin treatment for 30 minutes were set to 1 as depicted in Fig.3.1 - 3.6, which also represents the 0-hour time point. The interference of factors, other than the selected test compounds, on the activation status of Akt and total Akt levels were limited by culturing the cells in low glucose medium (5mM) and serum-starving plated cells for 24 hours prior to treatment with test compounds. It should be mentioned that the loading controls Hsp90 and EF1-α1 used for western blot analysis was not regulated by the treatment conditions (see Addendum C). Additionally, due to time constraints and difficulty experienced with the optimization of antibodies from multiple commercial sources, only two biological repeats were obtained for all time course experiments. For this reason, no statistical analysis was performed in Fig.3.1 – 3.6. A control with vehicle only was not included in the experiment, which is an important limitation and oversight of the experiment in examining the effectiveness of insulin to induce Akt phosphorylation and should be included in any future experiments. Nonetheless, insulin was shown to effectively induce Akt phosphorylation by another member in the research group (unpublished data390) using the exact same protocol. In addition, the effects of the test compounds alone were not included as the research question was to investigate how the test compounds affect the ability of insulin to induce Akt phosphorylation and most importantly FOXO1. Nonetheless, future studies should include how the test compounds affect basal Akt phosphorylation and total Akt levels.

3.2.1 Dex and F similarly affect the insulin-induced activation of Akt at Thr308

In BWTG3 cells, pre-treatment with 100nM Dex prior to insulin exposure appeared to reduce Thr308 phosphorylation of Akt at 24 hours as well as 48 hours as shown in Fig.3.1A. In comparison, treatment with 100nM Cort prior to insulin exposure reduced phosphorylation of Akt at Thr308 sooner at 2 hours to 24 hours. The ability of both GCs to inhibit insulin-induced phosphorylation of Akt was lost at later time points. Attenuation of insulin by Dex was lost at 72 hours (Fig.3.1A), whilst exposure to Cort for 48 hours and longer had no effect on insulin-induced Akt phosphorylation (Fig.3.1C). The total Akt levels in BWTG3 cells was not investigated due to time constraints imposed by the COVID-19 pandemic.
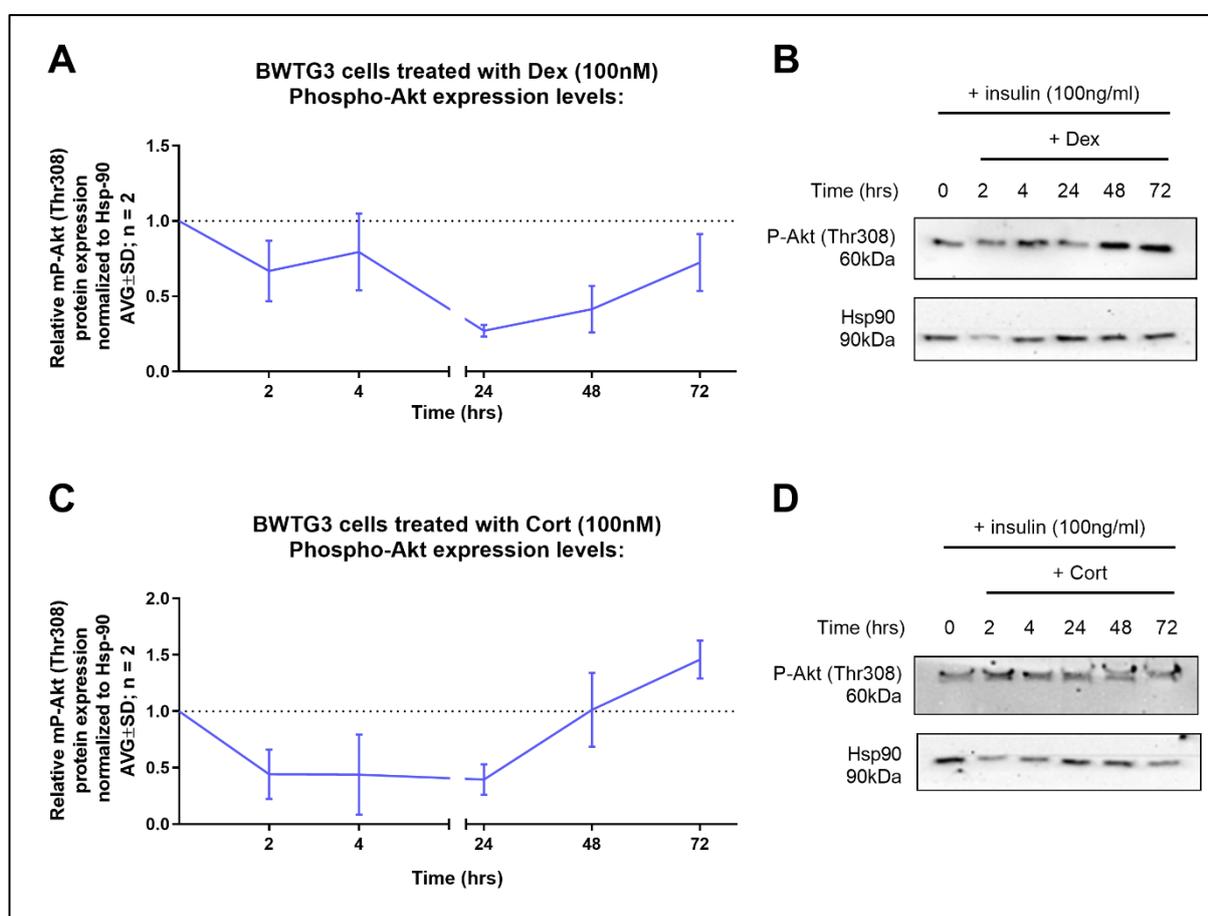
Figure 3.1. The GCs, Dex and Cort, decrease the insulin-induced phosphorylation of Akt at Thr308 in mouse hepatoma cells. BWTG3 cells were cultured and maintained in low glucose medium (5mM). After serum-starving for 24hrs, cells were treated with 100nM Dex **(A)**, or 100nM Cort **(C)**, for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. Phospho-Akt was normalized to the loading control Hsp90. Dex and Cort representative blots are shown in (**B & D**) respectively. Data shown represents two independent experiments. The GC treated time points were normalized to the vehicle control (set to 1) represented by the dotted line.

In comparison, HepG2 cells that were pre-treated with 100nM Dex before insulin stimulation, a reduction in Akt Thr308 phosphorylation was observed as early as 2 and 4 hours, and remained reduced at 24-, 48-, and 72 hours (Fig.3.2A). Furthermore, the attenuation of insulin-induced Akt phosphorylation by Dex was compared to total Akt protein levels (Fig.3.2A). Dex did not appear to have an effect on total Akt protein expression, indicating that the reduced insulin-induced activation of Akt *via* phosphorylation observed was not due to a decrease in total Akt protein levels, but rather due to Dex hindering the ability of insulin to activate Akt sufficiently. HepG2 cells were similarly pre-treated with 100nM F, where F showed a reduction in insulin-induced phosphorylation of Akt at all time points examined (Fig.3.3A). A slight increase in total Akt protein levels was observed when cells were pre-treated with F (Fig.3.3A). As with Dex, this suggests that F prevents insulin from sufficiently activating Akt *via* phosphorylation, which cannot be attributed to a decrease in total Akt protein expression.
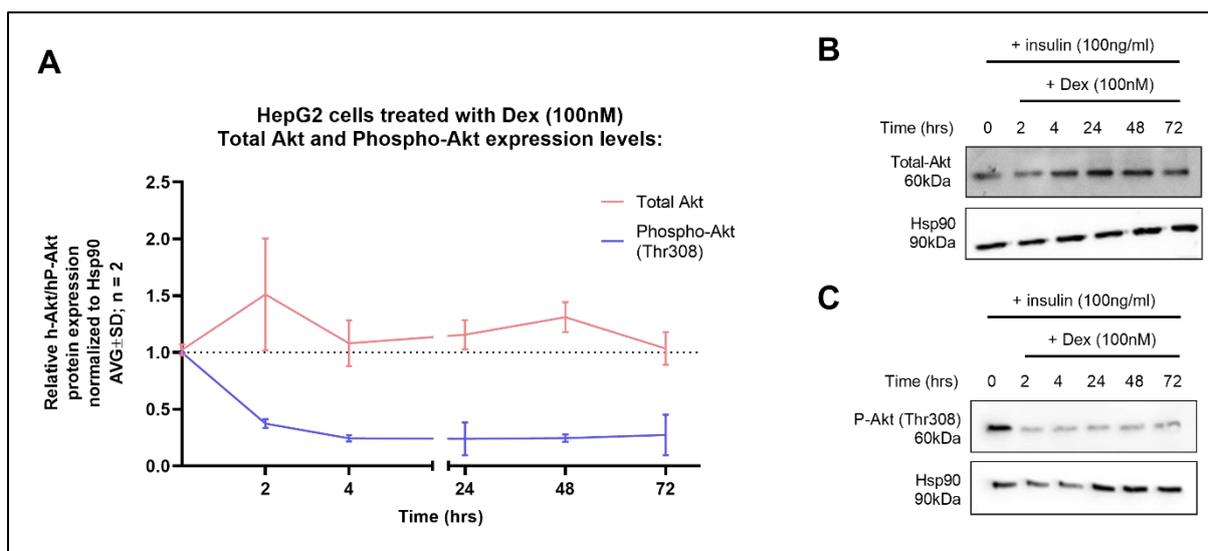
Figure 3.2. The exogenous GC Dex decreases insulin-induced Akt phosphorylation at Thr308 in human hepatoma cells. HepG2 cells were cultured and maintained in low glucose medium (5mM). After serum-starving for 24hrs, cells were treated with 100nM Dex for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. Phosphorylation of Akt at Thr308 and total Akt was normalized to the loading control Hsp90 **(A)**. Total Akt and Phospho-Akt representative blots are shown in **(B & C)** respectively. Data shown represents two independent experiments. The GC treated time points were normalized to the vehicle control (set to 1) represented by the dotted line.

In summation, both the exogenous and endogenous GCs appeared to attenuate the ability of insulin to activate Akt *via* phosphorylation at Thr308 in both hepatocyte cell line models. For Dex, the greatest reduction of insulin-induced Akt phosphorylation at Thr308 was observed at 24 hours in the BWTG3 cells, whilst in the HepG2 cells insulin-induced Akt phosphorylation was already inhibited by Dex at 2 hours pre-treatment and remained inhibited up to 72 hours. Unlike Dex, inhibition of Akt phosphorylation by Cort in the BWTG3 cells was observed sooner (2 hours) but was only sustained for 24 hours. Similarly, the endogenous GC, F, in the HepG2 cells reduced insulin-induced Akt phosphorylation at the earliest time point measured (2 hours), but in contrast to the observation made in the BWTG3 cells, remained inhibited up to 72 hours. Since insulin resistance is associated with a weakened ability of insulin to induce phosphorylation of Akt[417–421], it was decided that further experiments with the GCs would be performed by pre-treating cells for 24 hours prior to insulin exposure.
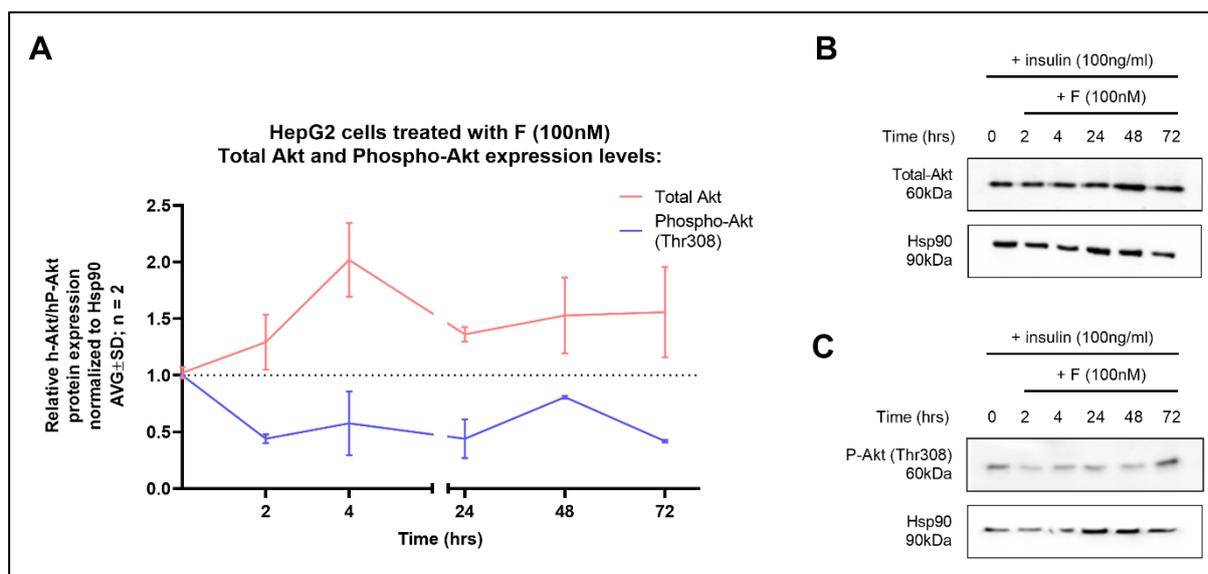
Figure 3.3. The endogenous GC F reduces insulin-induced phosphorylation of Akt at Thr308, in human hepatoma cells. HepG2 cells were cultured and maintained in low glucose medium (5mM). After serum-starving for 24hrs, cells were treated with 100nM F for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. Phosphorylation of Akt at Thr308 and Total Akt was normalized to the loading control Hsp90 **(A)**. Total Akt and Phospho-Akt representative blots are shown in **(B & C)** respectively. Data shown represents two independent experiments. The GC treated time points were normalized to the vehicle control (set to 1) represented by the dotted line.

3.2.2 TNF-α and IL-6 both regulate the insulin-induced activation of Akt at Thr308

To establish conditions an insulin resistant state (i.e., reduced insulin-induced Akt phosphorylation for the purposes of this MSc study) in the BWTG3 and HepG2 cells in response to inflammation, both cell lines were treated with 20ng/ml pro-inflammatory cytokines (TNF-α or IL-6) over time and the phosphorylation of Akt at Thr308 and total Akt was measured using western blot analysis.

BWTG3 cells pre-treated with TNF-α appeared to slightly reduce insulin-induced phosphorylation of Akt at Thr308 at 2- and 4 hours (Fig3.4A). Furthermore, the phosphorylation of Akt after 24 hours seemed to increase. Similarly, BWTG3 pre-treated with IL-6 also showed a small decrease in the ability of insulin to phosphorylate Akt for the first 4 hours (Fig.3.4C).
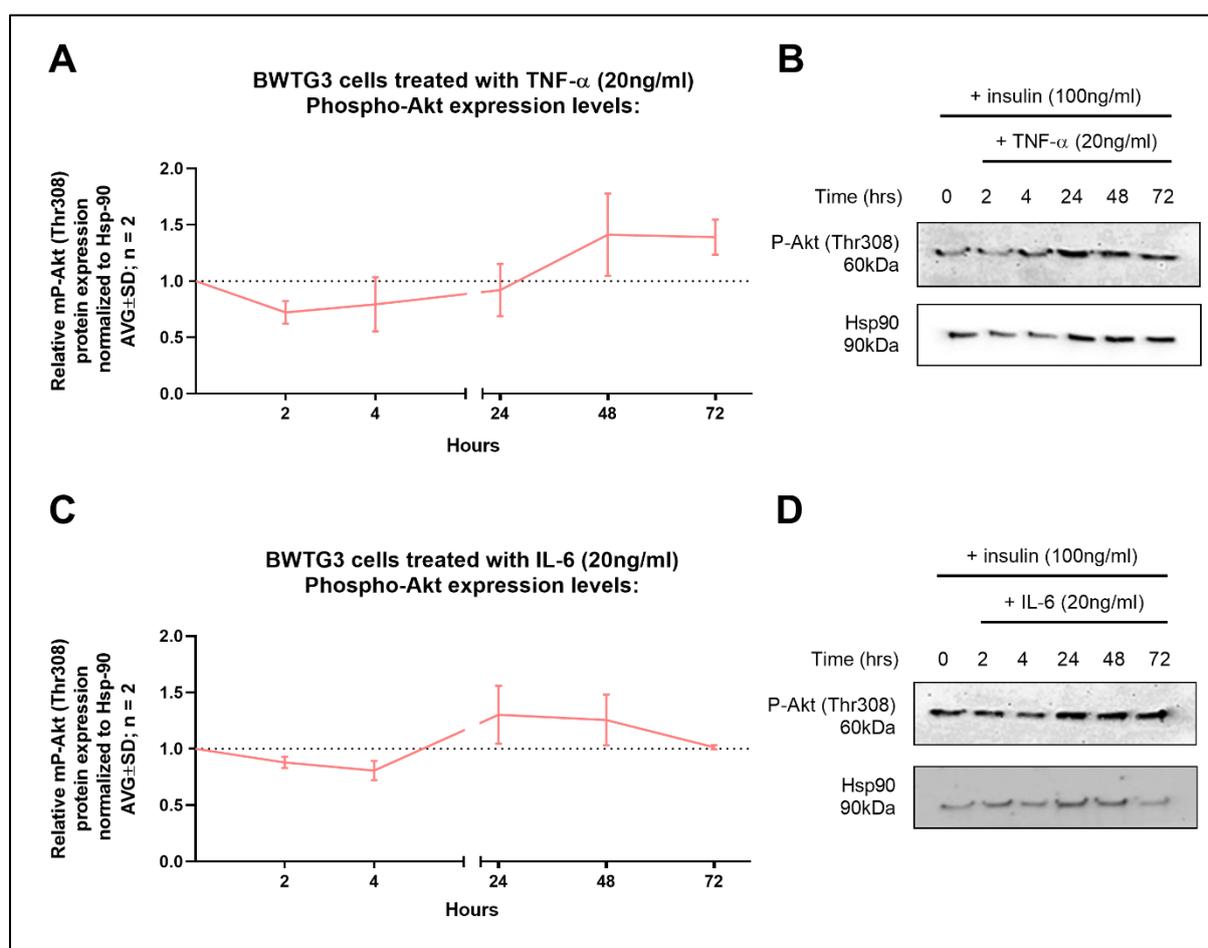
(Figure legend on next page).

Figure 3.4. The pro-inflammatory cytokines, TNF-α and IL-6, reduces insulin-induced phosphorylation of Akt at Thr308 at early time points in mouse hepatoma cells. BWTG3 cells were cultured and maintained in low glucose medium (5mM). After serum-starving for 24hrs, cells were treated with 20ng/ml TNF-α **(A)**, or 20ng/ml IL-6 **(C)**, for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. Phospho-Akt was normalized to the loading control Hsp90. TNF-α and IL-6 representative blots are shown in (**B & D**) respectively. Data shown represents two independent experiments. The cytokine treated time points were normalized to the vehicle control (set to 1) represented by the dotted line.

HepG2 cells exposed to TNF-α before insulin stimulation appeared to decrease insulin-induced Akt phosphorylation at 2- and 4 hours (Fig.3.5A). Inhibition of the insulin-induced Akt activation however, was short-lived, as at 24 hours TNF-α treatment resulted in the potentiation of the insulin-induced phosphorylation of Akt at Thr308, which gradually decreased over time to levels observed with insulin-only treatment. Pre-treatment with TNF-α did appear to increase total Akt levels at 24-, 48, and 72 hours in HepG2 cells, which could explain the increase in Akt phosphorylation observed at 24 hours (Fig.3.5A).

HepG2 cells were also subjected to IL-6 treatment prior to insulin exposure where 2- and 4 hours pre-treatment with IL-6 showed a reduction in insulin-induced Akt phosphorylation at Thr308 (Fig.3.6A). At 24 hours onwards, pre-treatment with IL-6 showed an increase the ability of insulin to phosphorylate Akt. Pre-treatment with IL-6 appeared to increase total Akt protein expression at 2 hours despite the reduction in Akt phosphorylation at the same time point. Total Akt protein expression remained unchanged at subsequent exposure times to IL-6 except for an apparent increase at 72 hours with the pro-inflammatory cytokine (Fig.3.6A).

To conclude, both inflammatory mediators were able to reduce the ability of insulin to activate Akt at Thr308 at 2- and 4 hours in the BWTG3 cells. In comparison, both inflammatory mediators reduced insulin-induced Akt phosphorylation at 4 hours in HepG2 cells. From these results it was concluded that the inhibition of insulin-induced Akt phosphorylation by the pro-inflammatory cytokines was best observed at 4 hours pre-treatment in both the BWTG3 and HepG2 cells, and all further experiments were performed using these conditions. In addition, it was also decided that all subsequent experiments will be performed using only the HepG2 cell line, as both the GC- and pro-inflammatory cytokine-induced insulin resistance states in the human hepatoma cell line was more pronounced.
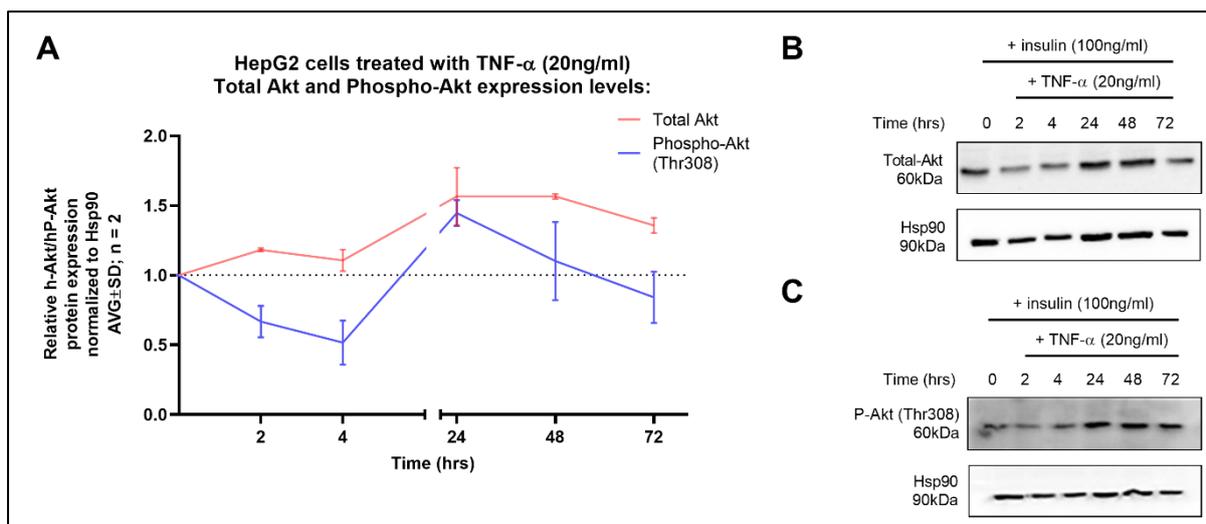
**Figure 3.5. The pro-inflammatory cytokine TNF-α can temporarily reduce insulin-induced phosphorylation of Akt at Thr308 in human hepatoma cells.** HepG2 cells were cultured and maintained in low glucose medium (5mM). After serum-starving for 24hrs, cells were treated with 20ng/ml TNF-α for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. Phosphorylation of Akt at Thr308 and Total Akt was normalized to the loading control Hsp90 **(A)**. Total Akt and Phospho-Akt representative blots are shown in **(B & C)** respectively. Data shown represents two independent experiments. The cytokine treated time points were normalized to the vehicle control (set to 1) represented by the dotted line.

Figure 3.6. The pro-inflammatory cytokine IL-6 can interfere with insulin's ability to phosphorylate Akt at Thr308 briefly in human hepatoma cells. HepG2 cells were cultured and maintained in low glucose medium (5mM). After serum-starving for 24hrs, cells were treated with 20ng/ml IL-6 for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. Phosphorylation of Akt at Thr308 and Total Akt was normalized to the loading control Hsp90 **(A)**. Total Akt and Phospho-Akt representative blots are shown in **(B & C)** respectively. Data shown represents two independent experiments. The cytokine treated time points were normalized to the vehicle control (set to 1) represented by the dotted line.

3.3 Exposure to stress and inflammation differentially regulate the mRNA expression of FOXO1

One of the key processes that is vital in glucose metabolism is gluconeogenesis, the pathway responsible for the *de novo* synthesis of glucose. Increased plasma glucose levels trigger the secretion of insulin, which in turn inhibits gluconeogenesis *via* the insulin signalling pathway so that homeostasis can be maintained. One mechanism through which gluconeogenesis can be halted is by inactivating the FOXO1 transcription factor *via* phosphorylation. As mentioned previously, FOXO1 transcriptionally regulates two key gluconeogenic enzymes, G6Pase and PEPCK. Changes in FOXO1 levels could directly influence gluconeogenesis and as a result, plasma glucose levels.

Insulin resistance is closely associated with elevated plasma glucose concentration[429–433]. Since both GCs and pro-inflammatory cytokines interfere with insulin signalling, it is of interest to investigate the effect stress and inflammation may have on the regulation of FOXO1. Thus, the aim of this experiment was to investigate the mRNA expression levels of the FOXO1 transcription factor in response to stress and inflammation in the human hepatoma cells. More specifically, FOXO1 mRNA expression levels in response to the GCs in the absence and presence of the pro-inflammatory cytokines were investigated.

The mRNA expression levels of FOXO1 were examined using qPCR. To allow correlation with the previous results, the treatment times used for GCs were 24 hours, and 4 hours for the pro-inflammatory cytokines, as in accordance with the previous section.

3.3.1 FOXO1 mRNA expression is differently regulated by the GCs alone as well as in combination with the pro-inflammatory cytokines

To determine the effects of stress and/or inflammation on FOXO1 mRNA expression, HepG2 cells were treated with Dex alone, or in combination with either TNF-α or IL-6. Insulin (100ng/ml) was added for 30min prior to cell lysis, except in the case of the vehicle control. The effect on the FOXO1 mRNA expression in response to insulin and the test compounds were compared to the vehicle control, which was set to 1 (represented by the dotted line).

Insulin alone had little effect on the mRNA expression of FOXO1. Upon the pre-treatment of Dex however, FOXO1 mRNA expression significantly increased ($p < 0.05$) by nearly 4-fold (Fig.3.7). The mRNA expression of FOXO1 also appeared to increase when HepG2 cells were pre-treated with TNF-α by approximately 2-fold, although not significantly (Fig.3.7A). Surprisingly, the mRNA expression of FOXO1 dramatically ($p < 0.001$) increased, approximately 6-fold, in response to co-treatment with Dex and TNF-α (Fig.3.7A). A slight but not significant ($p > 0.05$) upregulation of FOXO1 mRNA expression in response to IL-6 was observed, similar to TNF-α (Fig.3.7B). Unlike TNF-α however, IL-6 was unable to co-operatively upregulate FOXO1 mRNA expression with Dex. IL-6 rather significantly ($p < 0.01$) inhibited the Dex-mediated increase in FOXO1 mRNA levels (Fig.3.7B).
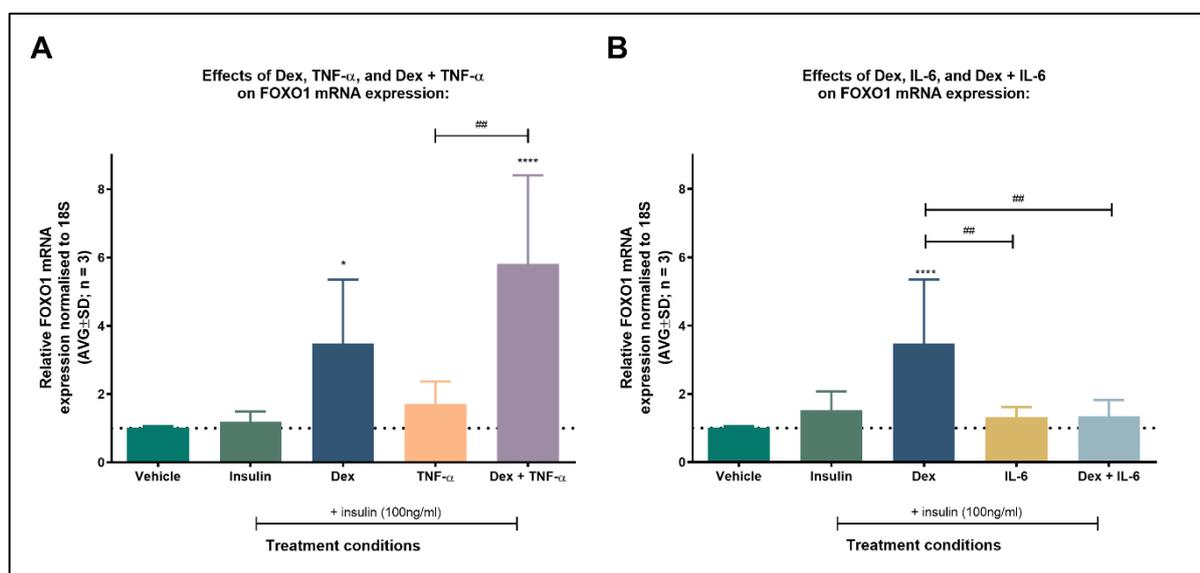
Figure 3.7. Dex and the pro-inflammatory cytokines TNF-α and IL-6, both alone and in combination, regulate FOXO1 mRNA expression differently in the human hepatoma cell line, HepG2. HepG2 cells were serum-starved for 24 hours followed by treatment with 100nM Dex for 24 hours **(A & B)**, 20ng/ml TNF-α for 4 hours **(A)**, or 20ng/ml IL-6 for 4 hours **(B)** before cell lysis. Cells were treated with 100ng/ml insulin 30min prior to cell lysis. The mRNA expression of the transcription factor FOXO1 was measured using qPCR and normalized relative to the housekeeping gene, 18S. Insulin's response was normalized to the vehicle (set to 1) which is represented as a dotted line. The response of Dex and the pro-inflammatory cytokines used were normalized to insulin. Data shown represents three independent experiments. Statistical analysis comparing insulin and the test compound treatments to the vehicle was performed using one-way ANOVA with Dunnett's multiple comparisons test (*: $p < 0.05$; *****: $p < 0.0001$). Additionally, analysis comparing treatment conditions relative to each other was performed using one-way ANOVA with Tukey's multiple comparisons test (##: $p < 0.01$).

Unlike the exogenous GC, equimolar F concentration did not upregulate FOXO1 mRNA expression in HepG2 cells (Fig. 3.8A). When HepG2 cells were treated with F in combination with TNF-α however, FOXO1 mRNA expression increased significantly ($p<0.01$), to 2.5-fold. This co-operative effect with TNF-α and F is similar to that observed with Dex and TNF-α. In contrast, a slight but non-significant ($p>0.05$) decrease in FOXO1 mRNA expression was observed when F was co-treated with IL-6 (Fig.3.8B).
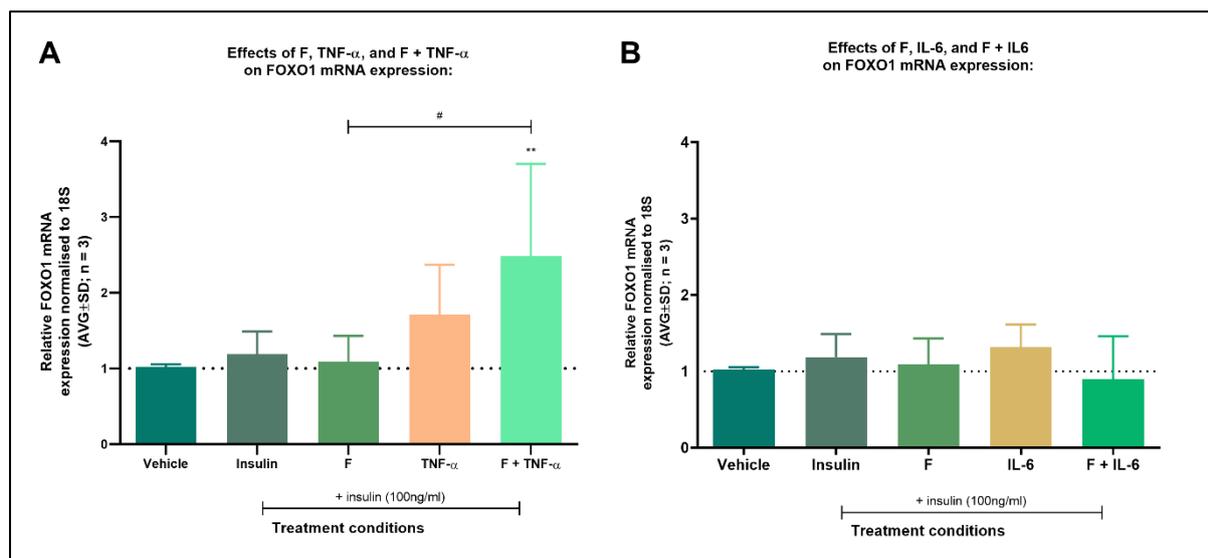
Figure 3.8. F and the pro-inflammatory cytokines TNF-α and IL-6, both alone and in combination, differentially regulate FOXO1 mRNA expression in the human hepatoma cell line, HepG2. HepG2 cells were serum-starved for 24 hours followed by treatment with 100nM F for 24 hours **(A & B)**, 20ng/ml TNF-α for 4 hours **(A)**, or 20ng/ml IL-6 for 4 hours **(B)** before cell lysis. Cells were treated with 100ng/ml insulin 30min prior to cell lysis. The mRNA expression of the transcription factor FOXO1 was measured using real-time PCR and normalized relative to the housekeeping gene, 18S. Insulin's response was normalized to the vehicle (set to 1) which is represented as a dotted line. The response of F and the pro-inflammatory cytokines used were normalized to insulin. Data shown represents three independent experiments. Statistical analysis comparing insulin and the test compound treatments to the vehicle was performed using one-way ANOVA with Dunnett's multiple comparisons test (**: $p<0.01$). Additionally, analysis comparing treatment conditions relative to each other was performed using one-way ANOVA with Tukey's multiple comparisons test (#: $p<0.05$).

In conclusion, only the exogenous GC, Dex was able to significantly upregulate the mRNA expression of FOXO1. An amplified increase in FOXO1 mRNA expression was observed in response to a combinatorial treatment with either GC and the cytokine TNF-α, compared to the relevant GC alone. The ability of Dex to increase FOXO1 mRNA expression in HepG2 cells when co-treated with IL-6 was hindered significantly, despite IL-6 alone slightly increasing FOXO1 mRNA expression. This result suggests that while both TNF-α and IL-6 are labelled as pro-inflammatory cytokines, they influence GC signalling differently *in vitro*.

3.3.2 Determining the effects stress and inflammation have on FOXO1 protein expression levels

The effects stress and inflammation have on the mRNA expression of FOXO1 give insight into whether such conditions affect the quantity of mRNA available to transcribe into a functional protein. It cannot be assumed however, that changes in mRNA expression result in similar changes in protein expression. Thus, a secondary aim of this study was to determine whether stress and inflammation influence FOXO1 protein expression in HepG2 cells using western blot analysis.

FOXO1 protein levels were measured over time in response to the GCs and the pro-inflammatory cytokines and compared to the vehicle-treated cells which was also exposed to 100ng/ml insulin for 30 minutes and set to 1, which represented the 0-hour time point. Due to difficulty experienced with the FOXO1 antibody, only 1 biological repeat could be obtained. Thus, no statistical analysis could be performed in Fig.3.9.

In the HepG2 cells, Dex appeared to have a biphasic effect on the FOXO1 protein expression as at earlier time points, 2- and 4 hours, FOXO1 protein levels decreased, which then increased to above basal at 24- and 48 hours to decrease again at 72 hours (Fig. 3.9A&B). Unlike Dex, treatment with the endogenous GC F displayed no biphasic effect. F at the earlier time points had minimal effect on the protein levels of FOXO1, whilst 24- and 48-hour exposure to F increased FOXO1 protein expression above 2-fold, which was slightly diminished at 72 hours although still greater than basal expression (Fig.3.9C&D). The pro-inflammatory cytokine TNF-α appeared to increase FOXO1 protein levels at all exposure times tested with the exception of 72 hours which had no effect (Fig. 3.9E&F). Conversely, short exposure times to IL-6 appeared to have no effect on FOXO1 protein expression whereas longer exposure to IL-6 led to a pronounced decrease in FOXO1 protein levels as approximately 2-fold less FOXO1 protein is observed when compared to basal FOXO1 protein levels (Fig. 3.9G&H).
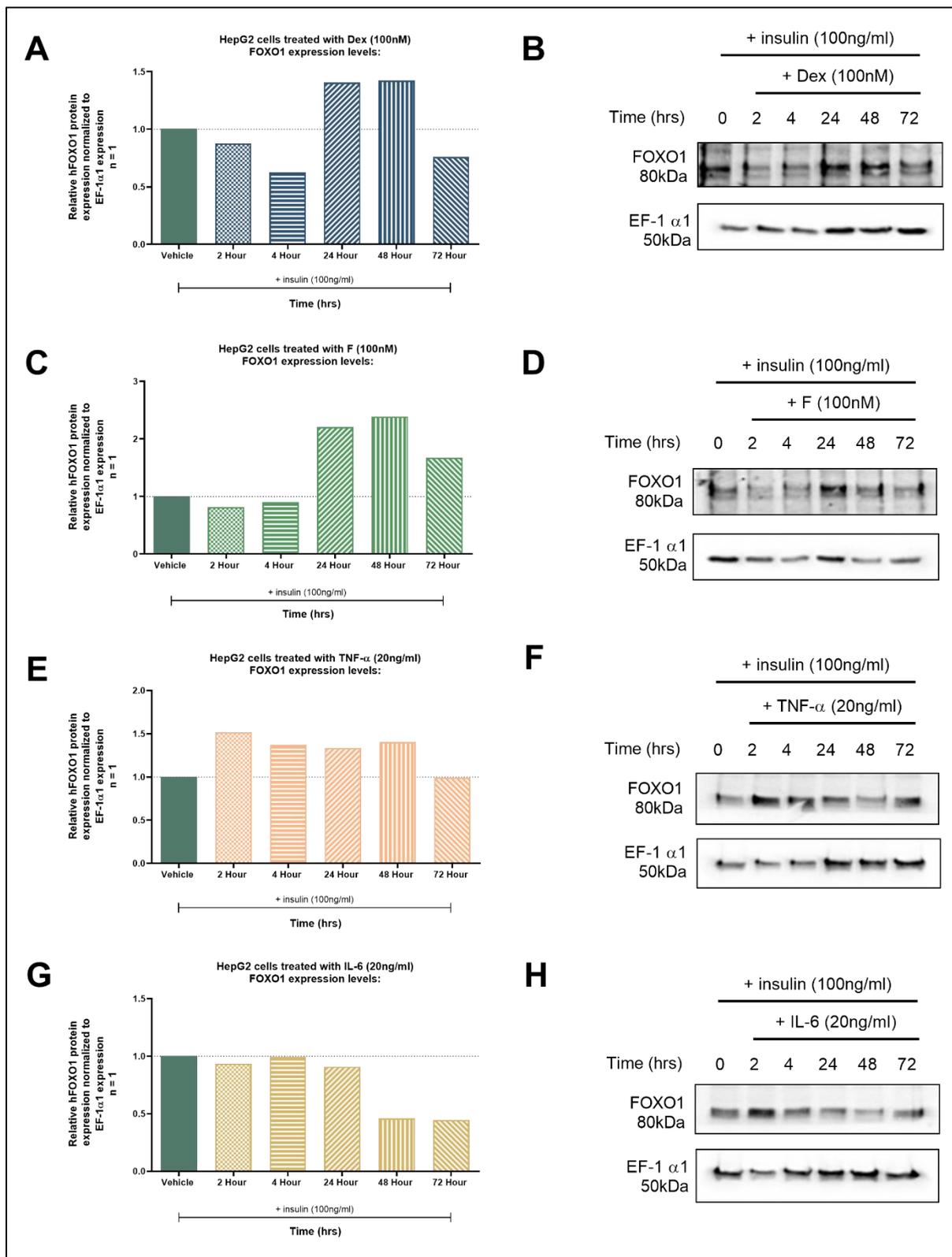
(Figure legend on next page).

Figure 3.9. FOXO1 protein expression in the human hepatoma cell line, HepG2, in response to the GCs, Dex and F, as well as the pro-inflammatory cytokines, TNF-α and IL-6. HepG2 cells were cultured and maintained in low glucose medium. After serum-starving for 24hrs, cells were treated with 100nM Dex **(A)**, 100nM F **(C)**, 20ng/ml TNF-α **(E)**, or 20ng/ml IL-6 **(G)** for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. FOXO1 was normalized to the loading control EF-1α1. The representative blots for Dex, F, TNF-α, and IL-6 are shown in **(B, D, F, and H),** respectively. Data shown represents one independent experiment. The test compound treated time points were normalized to the vehicle control (set to 1) represented by the dotted line.

3.4 Determining the influence stress and inflammation have on the promoter of FOXO1

The role of FOXO1 as a transcription factor in regulating numerous genes is well documented: FOXO1 can directly bind to the promoters of various genes, or indirectly *via* protein-protein interactions[138,187,321,434–437] . Little is known about the mechanisms that govern the gene transcription of FOXO1. Having shown that both GCs and pro-inflammatory cytokines affect FOXO1 mRNA and protein expression, the next aim of this study was to determine whether these affects could be attributed to the regulation of the *foxo1* promoter.

HepG2 cells were transiently transfected with a promoter reporter construct containing the mouse *foxo1* promoter (pGL3-mFOXO1wt-luc) before treatment with the GCs and pro-inflammatory cytokines, alone or in combination, prior to insulin exposure for 30min. The activity of the test compounds on the *foxo1* promoter was determined using a luciferase assay and measured in relative light units (RLU's). Insulin-only treatment had no significant effect ($p > 0.05$) on *foxo1* promoter activity.

3.4.1 Dose-dependent effect of the GCs on the *foxo1* promoter

To establish whether the GCs affect the promoter activity of *foxo1*, HepG2 cells, transiently transfected with a *foxo1* promoter containing luciferase reporter construct, were treated with 1nM, 10nM, and 100nM Dex or F.

Dex induced *foxo1* promoter activity at all concentrations tested (1nM and 10nM Dex ($p < 0.05$); 100nM Dex ($p < 0.0001$)) (Fig. 3.10A). The highest fold increase in promoter activity was observed with 100nM Dex (approximately 2.5-fold), which was significantly ($p < 0.0001$) higher than both the 1nM and 10nM treatments.

In comparison, the endogenous GC F at 1nM, significantly ($p < 0.05$) induced *foxo1* promoter activity by approximately 1.5-fold, similar to F at 10nM, although this was

found to be not significant. The highest fold increase was observed with 100nM F, which significantly (p<0.01) increased *foxo1* promoter activity by 1.8-fold (Fig.3.10B).

Both GCs induced the strongest *foxo1* promoter activity at 100nM, which was consequently selected for further *foxo1* promoter reporter experiments.
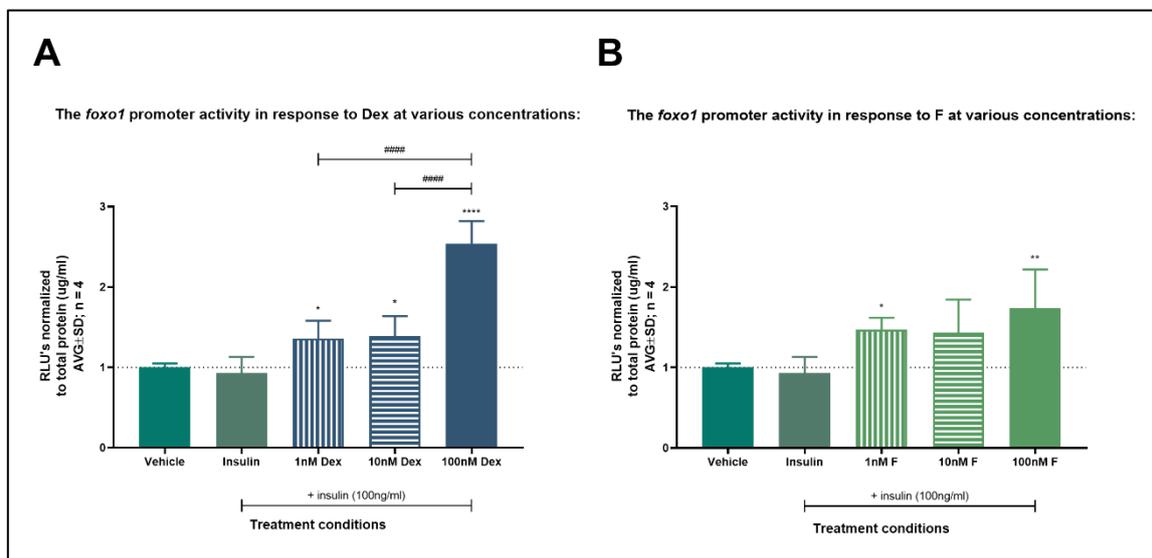
Figure 3.10. GC dose response to determine maximal activity on the promoter of FOXO1. HepG2 cells were transiently transfected with pGL3-mFOXO1wt-luc in a 10cm^2 dish. Following incubation for 24 hours, cells were replated into a 96 well plate. The next day, cells were treated with 1nM, 10nM, or 100nM Dex **(A)** or F **(B)** for 24 hours. Insulin was added 30 min before cell lysis. A luminometer was used to inject luciferin substrate and detect fluorescence, measured in RLU's. RLU's were normalized to protein concentration determined with a BCA assay. Insulin's response was normalized to the vehicle (set to 1) which is represented as a dotted line. The RLU's of Dex and F were normalized to insulin. Data shown represents four independent experiments. Statistical analysis comparing insulin and the test compound treatments to the vehicle was performed using one-way ANOVA with Dunnett's multiple comparisons test (*: p<0.05; **: p<0.01; ****: p<0.0001). Additionally, analysis comparing treatment conditions relative to each other was performed using one-way ANOVA with Tukey's multiple comparisons test (####: p<0.0001).

3.4.2 TNF-α and IL-6 differentially influence the GC-induced *foxo1* promoter activity

Having established that the GCs show an increase in the foxo1 promoter activity, we next wanted to determine whether the pro-inflammatory cytokines, TNF-α and IL-6, would affect the GC-induced response as observed with the FOXO1 mRNA expression.

As shown in Fig.3.10A, HepG2 cells transiently transfected with a *foxo1* promoter containing luciferase reporter construct (pGL3-mFOXO1wt-luc), treated with 100nM Dex resulted in a significant (p<0.0001) increase in *foxo1* promoter activity (Fig. 3.11A). In contrast, TNF-α did not significantly (p>0.05) affect the activity of the *foxo1*

promoter. Furthermore, TNF-α was unable to influence the Dex-induced increase in *foxo1* promoter activity (Fig.3.11A). Like TNF-α, IL-6 had no significant (p>0.05) influence on the promoter activity of *foxo1*, whilst it did significantly attenuate the Dex-mediated increase in *foxo1* promoter activity (p<0.0001). The attenuation by IL-6 of the Dex-induced *foxo1* promoter activity was however still significantly (p<0.0001) greater than basal activity (Fig.3.11B).

Like Dex, 100nM F-induced *foxo1* promoter activity was unaffected by TNF-α (Fig.3.12A) and significantly (p<0.01) inhibited by IL-6 (Fig.3.12B). IL-6 was able to decrease F-induced *foxo1* promoter activity to that of basal activity (Fig.3.12B).
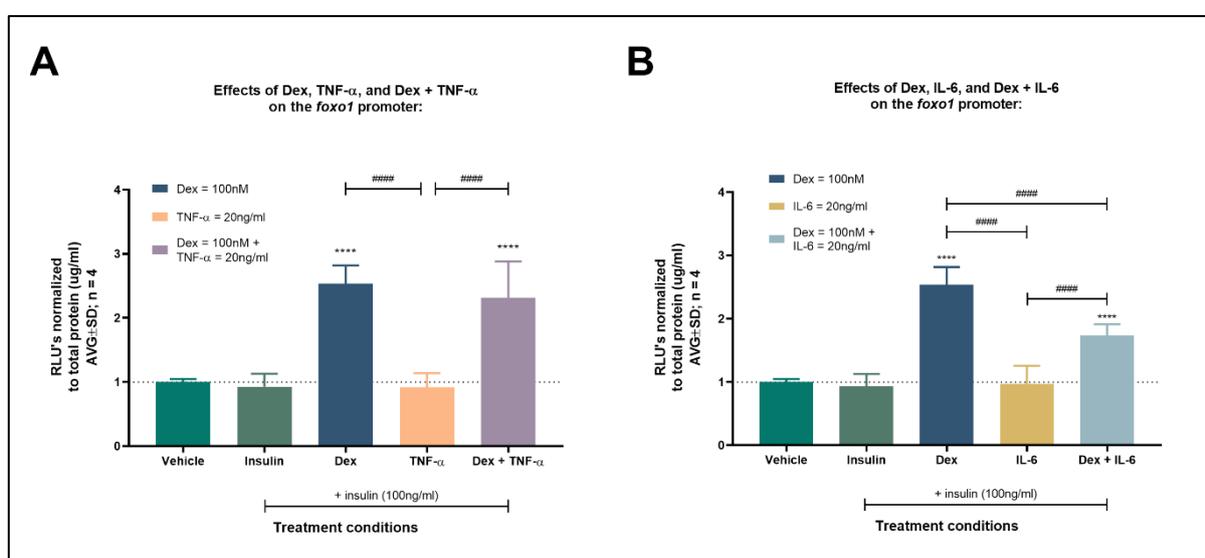
Figure 3.11. Pro-inflammatory cytokines, TNF-α and IL-6, differentially influence Dex-induced effects on the promoter of FOXO1. HepG2 cells were transiently transfected with a *foxo1* promoter reporter construct (pGL3-mFOXO1wt-luc) in a 10cm^2 dish. Twenty-four hours after transfection, cells were replated into a 96 well plate. The next day, cells were treated with 100nM Dex for 24 hours, 20ng/ml TNF-α for 4 hours, and a combination **(A)** or 20ng/ml IL-6 for 4 hours, and a combination **(B)**. Insulin was added 30 min before cell lysis. A luminometer was used to inject luciferin substrate and detect fluorescence, measured in RLU's. RLU's were normalized to protein concentration determined with a BCA assay. Insulin's response was normalized to the vehicle (set to 1) which is represented as a dotted line. The RLU's of the treatment conditions were normalized to insulin. Data shown represents four independent experiments. Statistical analysis comparing insulin and the test compound treatments to the vehicle was performed using one-way ANOVA with Dunnett's multiple comparisons test (****: p<0.0001). Additionally, analysis comparing treatment conditions relative to each other was performed using one-way ANOVA with Tukey's multiple comparisons test (####: p<0.0001).
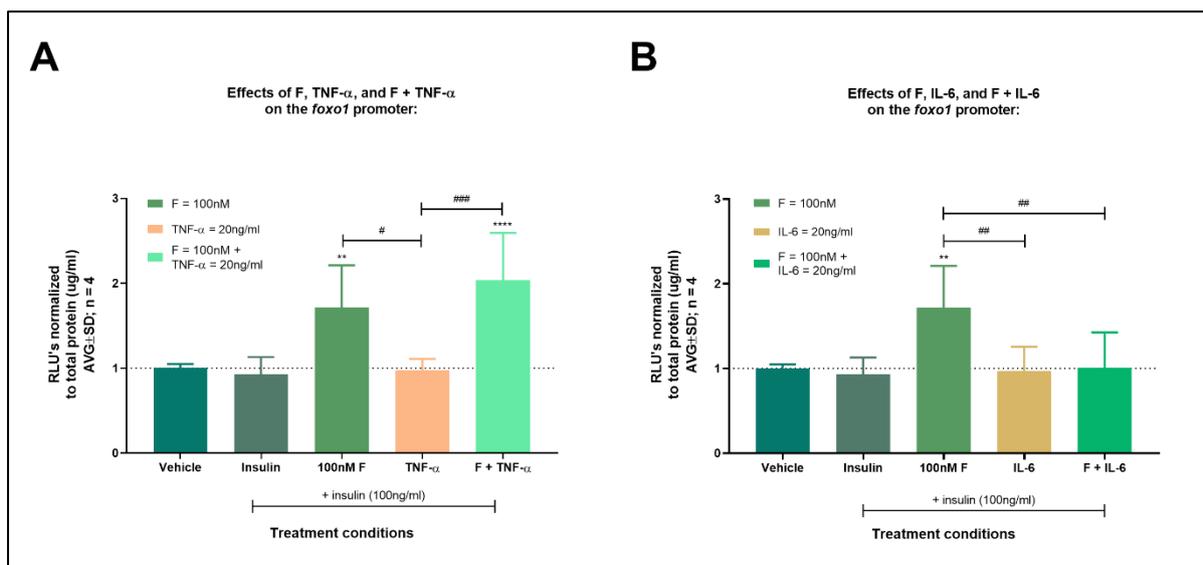
Figure 3.12. Pro-inflammatory cytokines, TNF-α and IL-6, differentially influence F-induced effects on the promoter of FOXO1. HepG2 cells were transiently transfected with a *foxo1* promoter reporter construct (pGL3-mFOXO1wt-luc) in a 10cm^2 dish. Twenty-four hours after transfection, cells were replated into a 96 well plate. The next day, cells were treated with 100nM F for 24 hours, 20ng/ml TNF-α for 4 hours, and a combination **(A)** or 20ng/ml IL-6 for 4 hours, and a combination **(B)**. Insulin was added 30 min before cell lysis. A luminometer was used to inject luciferin substrate and detect fluorescence, measured in RLU's. RLU's were normalized to protein concentration determined with a BCA assay. Insulin's response was normalized to the vehicle (set to 1) which is represented as a dotted line. The RLU's of the treatment conditions were normalized to insulin. Data shown represents four independent experiments. Statistical analysis comparing insulin and the test compound treatments to the vehicle was performed using one-way ANOVA with Dunnett's multiple comparisons test (**: $p<0.01$; ****: $p<0.0001$). Additionally, analysis comparing treatment conditions relative to each other was performed using one-way ANOVA with Tukey's multiple comparisons test (#: $p<0.05$; ##: $p<0.01$; ###: $p<0.001$).

To conclude, F alone can increase activity on the promoter of *foxo1*, much like Dex, whilst treatment with either one of the pro-inflammatory cytokines alone had no influence on the promoter. In addition, TNF-α had no influence on the ability of either GC to affect the *foxo1* promoter. IL-6, however, reduced *foxo1* promoter activity induced by both Dex and F. This finding indicates that while both TNF-α and IL-6 are considered pro-inflammatory cytokines that are implicated in insulin resistance, it cannot be assumed that these cytokines affect GCs in a similar manner.

3.4.4. TNF-α appears to have a direct effect on FOXO1 mRNA levels, whilst the GC Dex, and IL-6 requires *de novo* protein synthesis of an unknown regulatory protein

The promoter reporter assay (described in Section 3.4 above) gives a good indication whether a protein/molecule/test compound of interest has any influence on the

transcriptional activity of a gene. More specifically, the aim of the experiment above was to determine whether stress and/or inflammation had any influence on the promoter activity of the *foxo1* gene, which could explain the differential effects of the pro-inflammatory cytokines on the GC-induced increase in FOXO1 mRNA expression (Fig. 3.7 and Fig. 3.8). While the promoter reporter assay can identify changes in activity, thereby confirming or denying the promoter as a point of regulation by the GCs and pro-inflammatory cytokines, whether changes in transcriptional activity is due to a direct effect by the test compounds and their signalling proteins remains to be elucidated. It is for this reason that we wanted to determine whether *de novo* protein synthesis of a potential regulatory protein is responsible for the differential transcriptional activity observed in response to co-treatment of the GCs with the pro-inflammatory cytokines. Thus, HepG2 cells were treated with cycloheximide (CHX), a protein synthesis inhibitor to eliminate all indirect influences on FOXO1 mRNA expression.

CHX is a naturally occurring antibiotic produced by the bacterium *Streptomyces griseus*. It interferes with the translocation step in the synthesis of proteins, preventing the elongation step from occurring during translation[438,439]. As a result, any mechanism that requires the synthesis of proteins other than what is already synthesized in the cell, is inhibited, or greatly reduced.

The mRNA expression levels of FOXO1 were determined using qPCR. HepG2 cells were treated similarly as described in Section 3.3 with the exception of the addition of 1µM CHX 1 hour prior to treatment with the test compounds.

Upon the addition of CHX, the effects of Dex on the mRNA expression of FOXO1 diminished significantly ($p < 0.01$), as well as Dex in combination with TNF-α ($p < 0.0001$). TNF-α appeared to diminish mRNA expression, however the decrease was not significant (Fig.3.13A). Similarly, CHX was unable to significantly ($p > 0.05$) reduce the IL-6-mediated effect on FOXO1 mRNA expression (Fig.3.13B). A reduction was not seen in the Dex and IL-6 combination treatment either.

A The influence of CHX on FOXO1 mRNA expression levels in response to Dex, TNF-α, and Dex + TNF-α:

Insulin	+	+	+	+	+	+	+	+
Dex	-	-	+	+	-	-	+	+
TNF-α	-	-	-	-	+	+	+	+
CHX	-	+	-	+	-	+	-	+

B The influence of CHX on FOXO1 mRNA expression levels in response to Dex, IL-6, and Dex + IL-6:

Insulin	+	+	+	+	+	+	+	+
Dex	-	-	+	+	-	-	+	+
IL-6	-	-	-	-	+	+	+	+
CHX	-	+	-	+	-	+	-	+
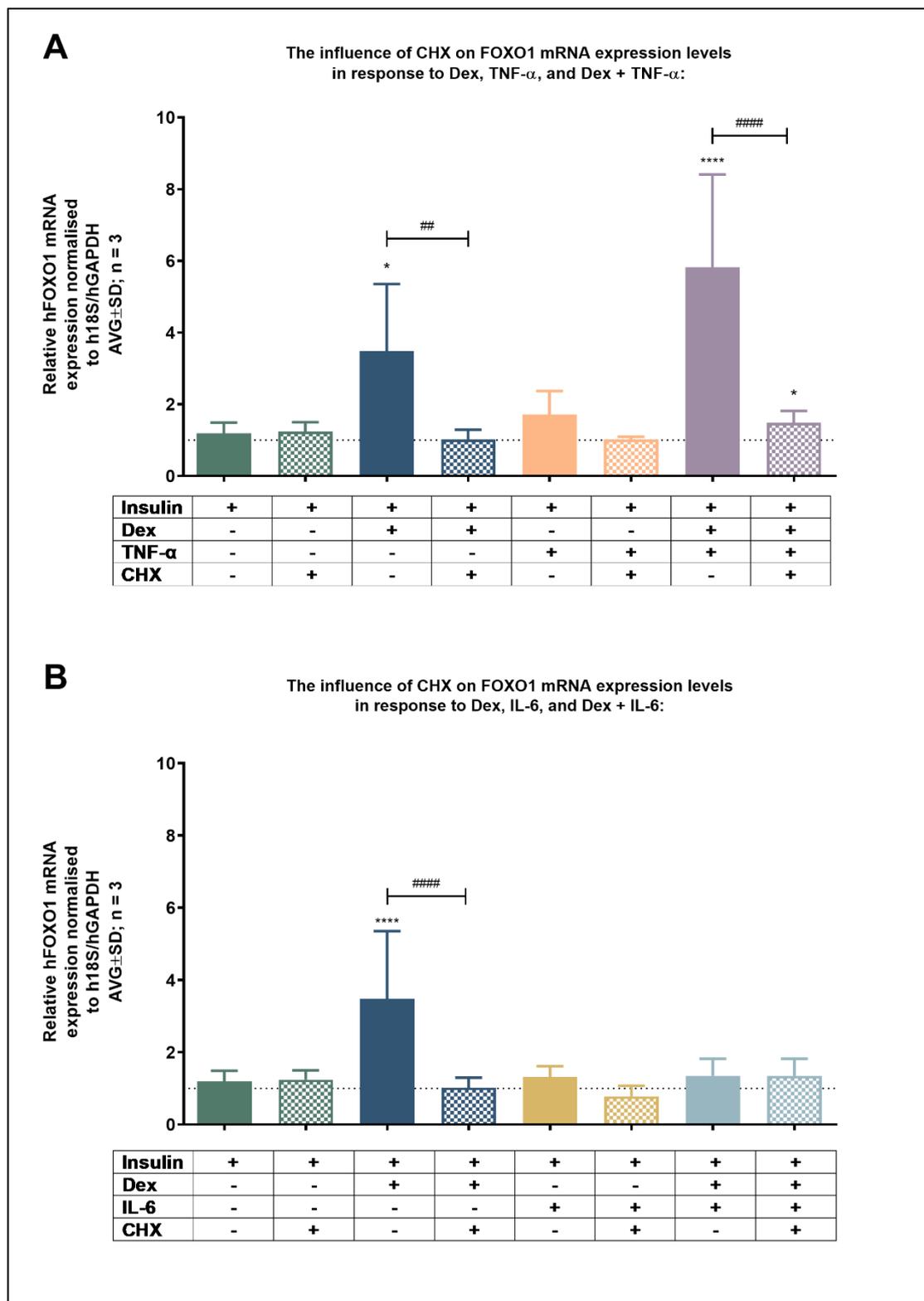
(Figure legend on next page).

Figure 3.13. CHX inhibits Dex-induced expression of the FOXO1 gene but seems to have differential influence on cytokine-induced expression of the FOXO1 gene in the human hepatoma cell line, HepG2. HepG2 cells were serum-starved for 24 hours followed by treatment with CHX for 1 hour before adding 100nM Dex for 24 hours **(A & B)**, 20ng/ml TNF-α for 4 hours **(A)**, or 20ng/ml IL-6 for 4 hours **(B)** before cell lysis. Cells were treated with 100ng/ml insulin 30min prior to cell lysis. The mRNA expression of the transcription factor FOXO1 was measured using qPCR and normalized relative to the housekeeping gene, 18S/GAPDH. Insulin's response was normalized to the vehicle (set to 1) which is represented as a dotted line. The response of the test compounds used was normalized to insulin. Data shown represents three independent experiments. Statistical analysis comparing insulin and the test compound treatments to the vehicle was performed using one-way ANOVA with Dunnett's multiple comparisons test (*: $p<0.05$; ****: $p<0.0001$). Additionally, analysis comparing each specific treatment conditions in the absence and presence of CHX two-way ANOVA with Bonferroni's multiple comparisons test (##: $p<0.01$; ####: $p<0.0001$) was done.

Upon the addition of CHX to F treatment, there was no significant difference in FOXO1 mRNA expression, (Fig.3.14A). As with Dex and TNF-α, CHX reduced the expression levels when F was treated in combination with TNF-α significantly ($p<0.05$) (Fig.3.14A). Interestingly, when CHX was added along with F and IL-6 combination treatments, there was a significant increase in FOXO1 mRNA expression ($p<0.05$).

In summary, these results suggest that the exogenous GC Dex appears to indirectly affect FOXO1 mRNA expression, possibly *via* the regulation of an unknown protein. Although significance could not be established, TNF-α and IL-6 also might indirectly regulate FOXO1 mRNA expression as a decrease to basal FOXO1 mRNA levels was observed in the presence of CHX.

A The influence of CHX on FOXO1 mRNA expression levels in response to F, TNF-α, and F + TNF-α:

Insulin	+	+	+	+	+	+	+	+
F	-	-	+	+	-	-	+	+
TNF-α	-	-	-	-	+	+	+	+
CHX	-	+	-	+	-	+	-	+

B The influence of CHX on FOXO1 mRNA expression levels in response to F, IL-6, and F + IL-6:

Insulin	+	+	+	+	+	+	+	+
F	-	-	+	+	-	-	+	+
IL-6	-	-	-	-	+	+	+	+
CHX	-	+	-	+	-	+	-	+

(Figure legend on next page).

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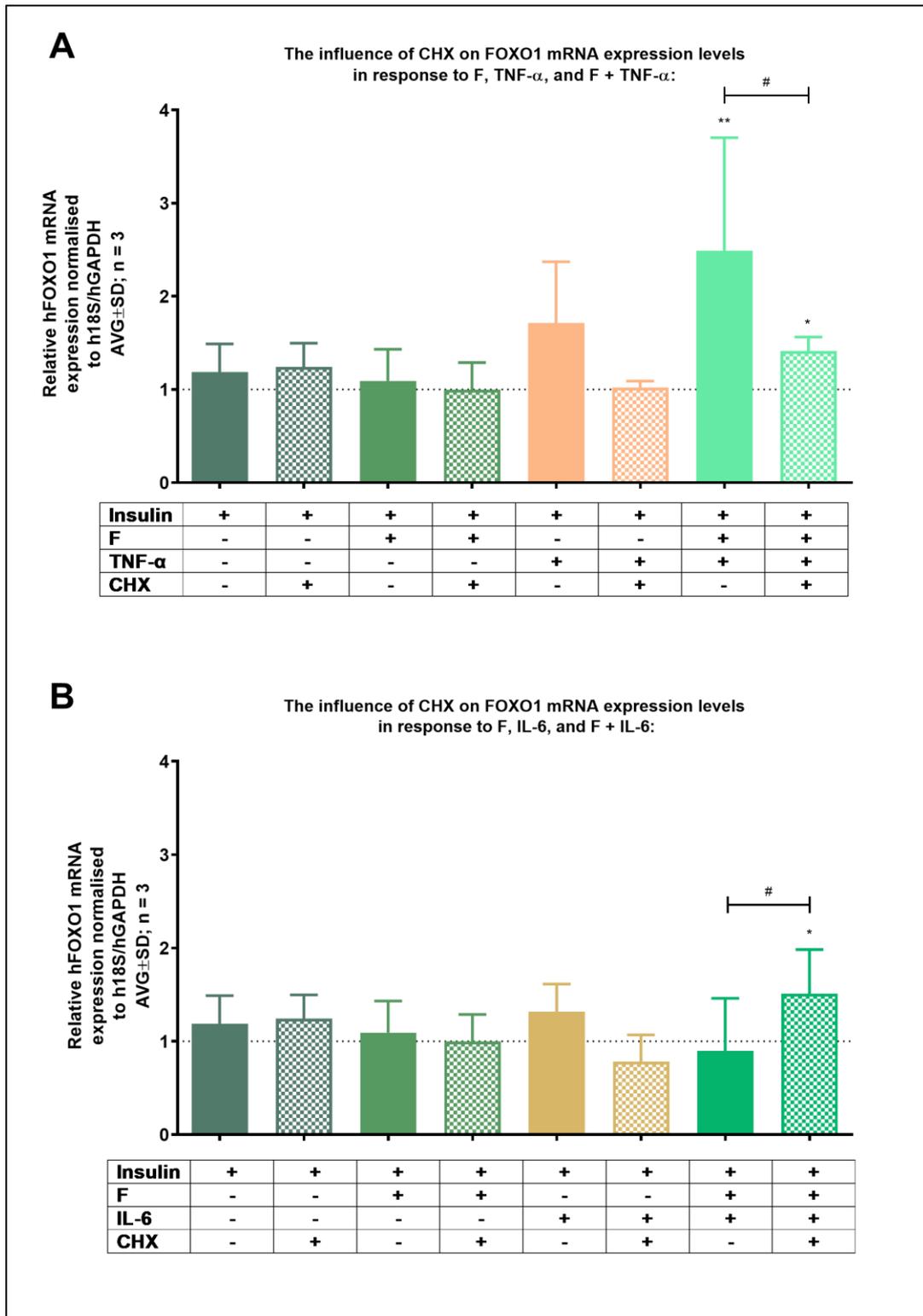
Figure 3.14. CHX appears to have differential effects on expression of the FOXO1 gene in the human hepatoma cell line, HepG2 when treated with F in combination with either TNF-α or IL-6. HepG2 cells were serum-starved for 24 hours followed by treatment with CHX for 1 hour before adding 100nM F for 24 hours **(A & B)**, 20ng/ml TNF-α for 4 hours **(A)**, or 20ng/ml IL-6 for 4 hours **(B)** before cell lysis. Cells were treated with 100ng/ml insulin 30min prior to cell lysis. The mRNA expression of the transcription factor FOXO1 was measured using qPCR and normalized relative to the housekeeping gene, 18S/GAPDH. Insulin's response was normalized to the vehicle (set to 1) which is represented as a dotted line. The response of the test compounds used was normalized to insulin. Data shown represents three independent experiments. Statistical analysis comparing insulin and the test compound treatments to the vehicle was performed using one-way ANOVA with Dunnett's multiple comparisons test (*: $p<0.05$; **: $p<0.01$). Additionally, analysis comparing treatment conditions relative to each other was performed using two-way ANOVA with Bonferroni's multiple comparisons test (#: $p<0.05$).

3.5 Stress and inflammation's effects on insulin induced post-transcriptional modifications of FOXO1

As mentioned previously, the FOXO1 transcription factor is inhibited *via* phosphorylation when the insulin signalling pathway has been stimulated by the presence of insulin. This halts the transcription of the PEPCK and G6Pase genes and inhibits gluconeogenesis. Furthermore, phosphorylated FOXO1 translocates from the nucleus to the cytoplasm where it gets tagged for degradation. It is therefore important not only to investigate the mRNA expression and protein level of FOXO1 in insulin resistance, but also the phosphorylation status of FOXO1 to determine its activity. states.

As we have established that stress and inflammation affect FOXO1 levels (Section 3.3), we next wanted to investigate whether GCs and pro-inflammatory cytokines interfere with the ability of insulin to inhibit FOXO1 *via* phosphorylation. Thus, the aim of this experiment was to investigate the phosphorylation status of FOXO1 in response to stress and inflammation in the human hepatoma cells as described in 3.2.

The activation of FOXO1 *via* phosphorylation was examined using western blot analysis. To analyse the effects of stress and inflammation on insulin-induced FOXO1 regulation, the effect of 100ng/ml insulin treatment for 30 minutes was set to 1 as depicted in Fig.3.15. The HepG2 cell line was cultured and treated as described previously. Only two biological repeats are shown for these experiments, thus no statistical analysis could be included for Fig.3.15.

As predicted, an increase in FOXO1 phosporylation was observed in the presence of insulin only (Fig.3.15). The pro-inflammatory cytokines TNF-α and IL-6 differentially affected insulin-induced phosphorylation of FOXO1. Pre-treatment with TNF-α prior to insulin exposure seemed to reduce FOXO1 phosphorylation to basal levels, different to insulin only treatment (Fig.3.15A&B). Meanwhile, IL-6 appeared to be able to attenuate insulin-induced FOXO1 phosphorylation when compared to the insulin control (Fig.3.15C&D). In fact, IL-6 reduced insulin-induced phosphorylation of FOXO1 to below basal levels (Fig.3.15C&D).

Like IL-6, pre-treatment with the exogenous GC Dex (100nM), reduced the insulin-induced FOXO1 phosphorylation to below that of basal phosphorylation levels (Fig.3.15A&C). Co-treatment with TNF-α however, increased FOXO1 phosphorylation to that of basal levels (Fig3.15A). Unlike with TNF-α, insulin-induced FOXO1 phosphorylation remained lower than basal levels when pre-treated with Dex and IL-6. In addition, co-treatment with Dex and IL-6 resulted in the phosphorylation of FOXO1 to be evidently lower than insulin only treatment (Fig3.15C).

The endogenous GC, F, when treated at an equimolar concentration as Dex appeared to reduce the ability of insulin to phosphorylate FOXO1 (Fig.3.15B&D). The addition of TNF-α had no effect on the ability of F to inhibit phosphorylation. The levels of FOXO1 phosphorylation remained unchanged and similar to basal levels regardless of insulin exposure (Fig.3.15B). Much like the effect of IL-6 with Dex, pre-treatment with F and IL-6 in combination was able to reduce FOXO1 phosphorylation compared to the insulin control. The attenuation of the insulin-induced FOXO1 phosphorylation resulted in levels below basal (Fig3.15D).

To conclude, both GCs were able to inhibit insulin-induced FOXO1 phosphorylation although to different degrees. The exogenous GC Dex was able to attenuate insulin-induced phosphorylation of FOXO1 to below basal levels whereas pre-treatment with F only resulted in FOXO1 phosphorylation to decrease back to basal. Similar to the GCs, the pro-inflammatory cytokines both decreased insulin-mediated FOXO1 phosphorylation albeit to different extents. IL-6 was able to decrease FOXO1 phosphorylation to below basal, whilst TNF-α only slightly decreased the insulin-induced phosphorylation of FOXO1 to approximately basal levels. Furthermore, IL-6 in the presence of either GC seemingly hindered the ability of insulin to phosphorylate

FOXO1. These results further contribute to the knowledge that treatment with the GCs and the pro-inflammatory cytokines induced an insulin-resistant state described in Section 3.2., affecting downstream insulin signalling mediated by the effector Akt.
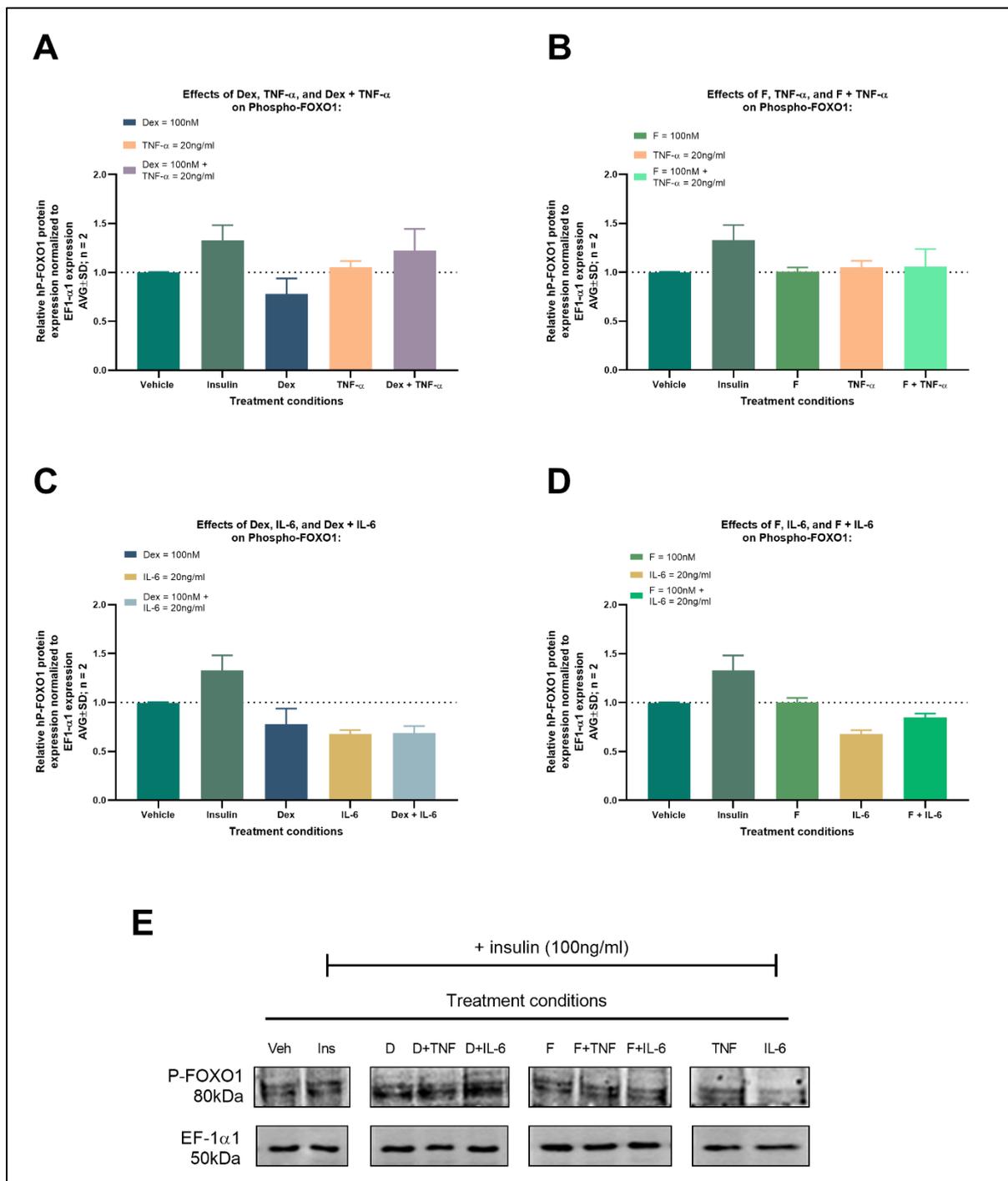
(Figure legend on next page).

Figure 3.15. F and TNF-α inhibit insulin's ability to phosphorylate FOXO1, while Dex and IL-6 decrease FOXO1 phosphorylation to below basal levels. HepG2 cells were cultured and maintained in low glucose medium (5mM). After serum-starving for 24hrs, cells were treated with 100nM Dex **(A & C)**, or 100nM F **(B & D)**, for 24hrs, or 20ng/ml TNF-α **(A & B)**, or 20ng/ml IL-6 **(C & D)**, for 4hrs, alone or in combination, before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. Phospho-FOXO1 was normalized to the loading control EF-1α1. Representative blots are shown in (**E**). Data shown represents two independent experiments. The treatment conditions were normalized to the vehicle control (set to 1) represented by the dotted line.

Chapter 4: Discussion and Conclusions

4.1 Introduction

T2DM affects an estimated 463 million people worldwide and is expected to reach 700 million people by the year 2045[1]. Furthermore, T2DM is the second highest cause of death in South Africa[1,5]. Insulin resistance is commonly associated with the development of T2DM, and it is therefore vital that the molecular mechanisms that contribute to insulin resistance is explored. Insulin resistance is generally described as the inability of insulin's target tissues (i.e., liver, skeletal muscle, and adipose tissue) to respond optimally to normal insulin concentrations[91,92]. This results in muted insulin signalling in these tissues, and the inability to halt gluconeogenesis[60,137]. The subsequent rise in blood glucose is characteristic of hyperglycaemia observed in T2DM. The effects described occur because of dysregulation in the activation or inhibition of key proteins involved in insulin signalling and glucose metabolism, including Akt and FOXO1.

Obesity is known to play a substantial role in the development of insulin resistance and is often associated with the presence of low-grade chronic inflammation[217–219]. Interestingly, chronic inflammation is also a contributor to the development of insulin resistance. The potential linking factor between obesity, chronic inflammation, and insulin resistance, is the increased secretion of pro-inflammatory cytokines from adipocytes, and the elevated serum levels of pro-inflammatory cytokines[213,284]. TNF-α and IL-6 specifically, are known to be secreted by adipocytes and are elevated in insulin resistant states[254,289,290]. Furthermore, pro-inflammatory cytokines stimulate the HPA axis to trigger the stress response, resulting in the secretion of endogenous GCs[331,333]. GCs have many roles in maintaining metabolic pathways including glucose metabolism. Chronic stress is thus also associated with an increased risk for developing insulin resistance[302].

Despite the link between inflammation and the stress response, little research is available on their combined effect on the progression of insulin resistance. The liver is the main site for gluconeogenesis and therefore plays a big part in the development of hyperglycaemia and T2DM. The FOXO1 transcription factor regulates gluconeogenesis in the liver by transcribing genes of two key enzymes required for

gluconeogenesis, namely PEPCK and G6Pase[164,187]. Furthermore, FOXO1 remains constitutively active in insulin resistant states. Thus, the focus of this study was to determine whether stress and inflammation, represented by GCs and pro-inflammatory cytokines, could induce hepatic insulin resistance, and whether this in turn effects FOXO1 regulation and activity. The activation of Akt, a key protein in insulin signalling responsible for the phosphorylation of FOXO1 was investigated to determine whether stress and inflammation could induce insulin resistance in a hepatocyte cell model. The regulation and activity of FOXO1 was then explored to understand its involvement in contributing to insulin resistance.

4.2 Establishing an insulin resistant state

In insulin resistant states, the ability of insulin to stimulate the insulin signalling pathway is attenuated, preventing the activation of downstream signalling proteins, as described in Chapter 1[93,440,441]. Diminished Akt phosphorylation, a central protein in insulin signalling, in response to insulin is often used as an indicator for insulin resistance[418,441]. While there are several other proteins in the insulin signalling pathway that have been implicated in insulin resistance, for the purpose of this study insulin resistance induced by either GCs or cytokines was presumed when insulin-induced activation of Akt via phosphorylation was impaired. Additionally, the activated Akt is directly responsible for FOXO1 phosphorylation, making it a key node in insulin signalling and FOXO1 regulation (Chapter 1)[16,21]. Furthermore, preliminary data from our research group showed that insulin-induced downregulation of G6Pase mRNA expression, a key gluconeogenic enzyme, was attenuated in the presence of the selected test compounds used in this study (unpublished data; Addendum D), which occurs downstream of Akt activation. Thus, the aim of the first part of the study was to investigate whether the GCs or pro-inflammatory cytokines could induce insulin resistance in two liver cell lines, namely HepG2 and BWTG3. In Chapter 3 it is shown that the selected test compounds varied in their ability to alter the activation status of Akt in a time-dependent manner, implying changes to downstream proteins as a result. This section will focus on how this result compares to literature and how this finding led to the subsequent aims.

Dex appeared to be able to attenuate insulin-induced phosphorylation in both the HepG2 and BWTG3 cells to a similar extent, although the HepG2 cell line appeared more responsive and attenuated insulin signalling as early as 2 hours compared to the

BWTG3 showing attenuation at 24 hours (Figure 3.1A & Figure 3.2A). The Dex-mediated inhibition of Akt phosphorylation at Thr308 agrees with the literature. Buren *et al.* have reported that Dex inhibits insulin-induced Thr308 phosphorylation of Akt in rat muscle cells and adipocytes[442]. In addition, Feng *et al.* found that Dex-treated rat liver cells results in a 64% reduction in insulin-induced Thr308 Akt phosphorylation[443]. It is important to note however, that full activation of Akt requires both Thr308 and Ser473 phosphorylation. Some studies do show Dex inhibits insulin-induced Ser473 phosphorylation of Akt[442,444]. It would therefore be interesting to expand the current study by determining whether Dex would also inhibit insulin-induced Akt phosphorylation at Ser473 in liver cells in the current model system.

Similar to Dex, the endogenous GCs, F and Cort, was able to inhibit the activation of Akt in their respective cell lines (Figure 3.1C & Figure 3.3A). Cort-mediated attenuation of insulin-induced Akt activation *via* phosphorylation was however short-lived, as at 48 hours and longer no inhibition was observed (Figure 3.1C). This could be explained by the shorter half-life of endogenous GCs that are readily metabolized in the liver, as mentioned in Chapter 1[445–447]. Unlike the BWTG3 cell line however, the endogenous F in HepG2 cells could inhibit insulin-induced activation of Akt for a longer period of time, further implying that the HepG2 cell line may be a more suitable cell model to use to study the effects of GCs on insulin signalling (Figure 3.3). Oddly, the influence of endogenous GCs on insulin signalling is contradictory. Park *et al.* demonstrated that Cort decreases Akt phosphorylation in rat muscle[448], whilst in contrast Molnar *et al.* observed an increase in Akt phosphorylation in the presence of Cort in rat muscle[449]. It should be noted however, that the increase in Akt phosphorylation observed by Molnar and colleagues is in the absence of insulin, whilst Park *et al.* observed a decrease in insulin-induced Akt phosphorylation by Cort. This would suggest that the effects of GCs on Akt activation is dependent on the presence of insulin.

Studies investigating the effects of GCs on insulin signalling in the liver however is lacking. This is probably due to GC-induced insulin resistance mainly being attributed to the direct effect of GCs on gluconeogenesis i.e., transcriptional regulation of PEPCK and G6Pase. Few studies investigate whether GCs negatively impact insulin signalling directly *via* targeting its multiple signalling nodes in the liver and thereby contributing to a hepatic insulin resistant state. This highlights the need for further research in stress-induced hepatic insulin resistance.

The pro-inflammatory cytokines appeared to be able to attenuate phosphorylation of Akt, albeit minimally (Figures 3.4, 3.5, and 3.6). Again, HepG2 cells were more responsive to treatment with either one of the cytokines, compared to the BWTG3 cell line. In addition, the attenuation of insulin-induced phosphorylation by both pro-inflammatory cytokines was short-lived and was only observed for the first 4 hours, after which Akt phosphorylation was induced in the presence of insulin in both hepatoma cell lines (Figures 3.4, 3.5, and 3.6). Cytokines are known to have a short half-life[450–452]. This could explain the short-term inhibition of insulin-induced phosphorylation of Akt seen in this study. It is important to distinguish differences in pre-treating the cells with pro-inflammatory cytokines verses a chronic inflammatory state *in vivo*. In a chronic inflammatory state, pro-inflammatory cytokines are constantly being secreted by various cell and tissue types, resulting in constant stimulation of their respective receptors as discussed in Chapter 1. This implies that insulin signalling is perturbed continuously without the possibility for insulin to activate Akt fully, unlike what was observed with cells pre-treated with cytokines for 24 hours or longer in this study. Nonetheless, several studies show that TNF-α induces insulin resistance in the liver[453–456]. One study in particular demonstrated using HepG2 cells that insulin-induced Akt phosphorylation is attenuated in response to TNF-α treatment for 48 hours[375], which was not seen in the current study at 48 hours TNF-α exposure (Figure 3.5). These differences could be due to different culturing and experimental conditions. For example, HepG2 cells are traditionally maintained in culture medium containing 25mM glucose, which we[390] and others[457] have found to influence the sensitivity of the insulin signalling pathway to insulin. HepG2 cells grown in culture medium containing 25mM glucose are less sensitive to insulin-induced phosphorylation of the insulin receptor and Akt[390]. Also, Leontieva et al. eloquently showed that a more pronounced Akt phosphorylation was observed in C2C12 cells cultured in 5.5mM glucose vs 25mM glucose[457]. They in fact considered C2C12 cells cultured in 25mM glucose to be insulin resistant. Unfortunately, Miao *et al.* did not indicate the concentration of glucose used in their culture medium. Differences in response to insulin would be expected with differences in glucose concentration. In addition, Miao and collegues used 300 nM insulin for 10 min and 10 ng/ml TNF-α, while the current study used 100 ng/ml insulin for 30 min and 20 ng/ml TNF-α, which could also explain the differences between the two studies.

TNF-α also appears to be responsible for the phosphorylation of IRS at Ser307, resulting in the inability to phosphorylate downstream targets like Akt (discussed in Chapter 1)[458,459,375]. Like TNF-α, IL-6 has also been implicated in hepatic insulin resistance[289,460,461]. Klover *et al.* found that IL-6 depletion in mouse hepatocytes results in an increase in Akt phosphorylation, implying IL-6 inhibits insulin-induced Akt activation[460].

In summation, both GCs and pro-inflammatory cytokines attenuate hepatic insulin signalling, at least in terms of Akt phosphorylation. While this study supports the idea that GCs and cytokines induce hepatic insulin resistance, it is important to note that there are many factors that can contribute to hepatic insulin resistance, including other insulin responsive tissues. This study would require additional biological repeats to determine the significance of these results and therefore the conclusions drawn from these results are preliminary. In addition, the error between the two repeats could be attributed to technical errors such as unequal amount of protein between samples due to poor pipetting. A limitation to the study is how the test compounds in the absence of insulin affect Akt phosphorylation and total protein expression. It should be noted however, that no basal Akt phosphorylation of Thr308 was observed in the liver cells of both *in vitro*[390] and *in vivo*[462] models, thus effects on Akt Thr308 phosphorylation by the test compounds in the absence of a stimulant would be difficult to detect. Nevertheless, the main focus of this study was to determine whether stress- and inflammation-induced hepatic insulin resistance (i.e., a state where also insulin-induced Akt phosphorylation is attenuated) had any effect on the FOXO1 transcription factor, a key regulatory protein in gluconeogenesis.

4.3 Stress and inflammation differentially regulate FOXO1 mRNA and protein expression

The FOXO1 transcription factor plays an important role in glucose metabolism, as discussed in Chapter 1. In insulin resistant states, FOXO1 remains active, due to evidence of increased PEPCK and G6Pase levels, resulting in an increase in glucose production[60,138,139]. The FOXO1 transcription factor can be regulated on one or several ways that affect how much FOXO1 is present as well as the activity of FOXO1 at a given time (Figure 4.1). As discussed in Chapter 1, the promoter of FOXO1 contains response elements through which transcription factors can regulate the transcription

of the *foxo1* gene. This in turn influences how much FOXO1 mRNA is available for translation. Furthermore, mRNA stability could be regulated, affecting the half-life of FOXO1 mRNA, resulting in changes to FOXO1 protein levels. It is also of importance to note that the FOXO1 protein is tightly regulated by several post-translational modifications, affecting its activity. These include phosphorylation, which results in the degradation of FOXO1. Despite FOXO1 being a key node in insulin signalling, few studies focus on the dysregulation of FOXO1 at one or more of its regulatory sites (Figure 4.1) in an insulin resistant state. Thus, this study aimed to determine if stress- and inflammation could regulate FOXO1 at several regulatory nodes. In Chapter 3 we show that the GCs and pro-inflammatory cytokines regulated FOXO1 expression differently, emphasizing the complexity of cross-talk between stress and inflammation. The possibilities in differential regulation of FOXO1 by these test compounds will be discussed below.
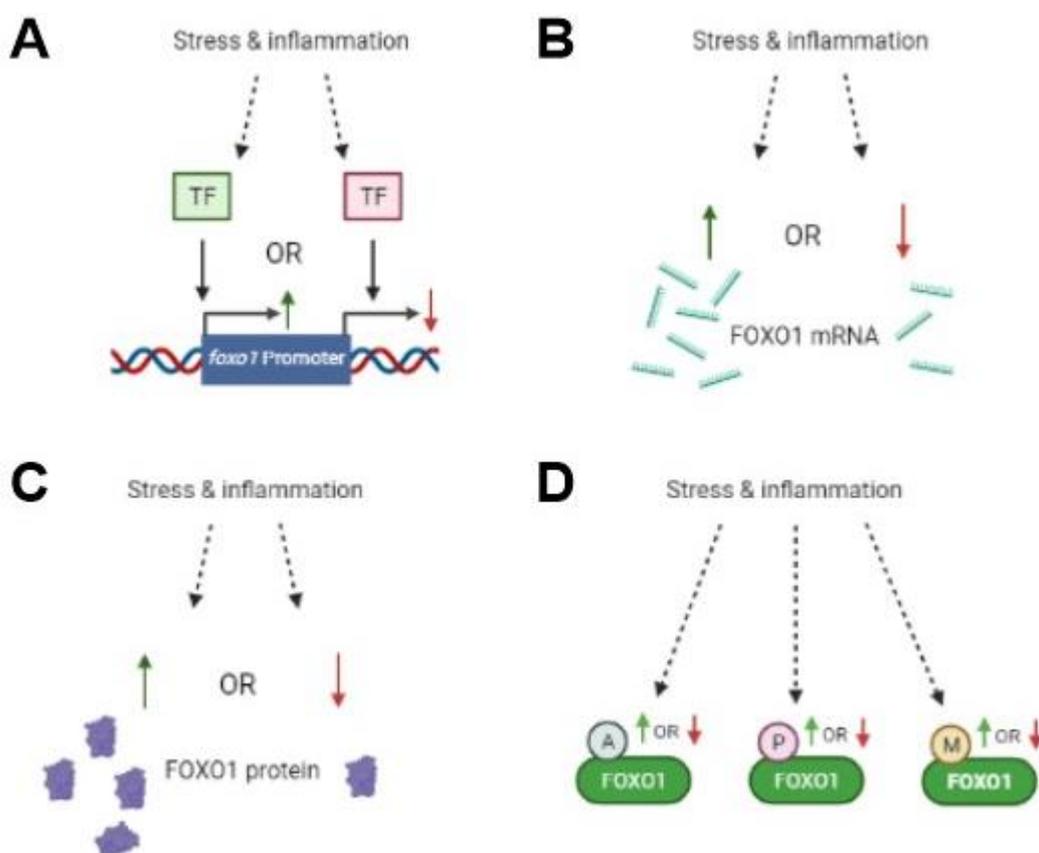
Figure 4.1. Schematic illustration of possible regulatory sites stress, and inflammation could influence the regulation of FOXO1. As with other proteins, FOXO1 can be regulated on one or more levels. Stress and inflammation could regulate the transcription of the *foxo1* gene through activity of transcription factors (TF) on the promoter of FOXO1 **(A)**. This in turn could influence the levels of FOXO1 mRNA available **(B)**. Furthermore, stress and inflammation could influence FOXO1 mRNA stability or half-life. Changes in FOXO1 mRNA could influence the amount of FOXO1 protein present in cells **(C)**. Stress and inflammation could also influence the post-translational modifications, such as acetylation (A), phosphorylation (P), and/or methylation (M), on FOXO1, determining its activity in cells **(D)**. Image drawn in Biorender.

HepG2 cells treated with Dex significantly upregulated FOXO1 mRNA expression (Figure 3.7) summarized in Table 4.1. This finding is consistent with both *in vitro* and *in vivo* studies in previous literature[174,321,322,372]. Interestingly, treatment with the endogenous GC, F, had no significant effect on the mRNA expression of FOXO1 (Figure 3.8) (Table 4.1). Contrary to this result, Cattaneo *et al.* described a 4-fold increase in FOXO1 mRNA expression induced by F in hippocampal stem cells[371]. It is important to mention that FOXO1 function varies across tissue types, and therefore could explain differences in its expression across cell types. In addition, the liver is the main site for F metabolism as mentioned previously, presenting the possibility of reduced effects of F seen on mRNA expression of FOXO1 in the liver. In addition, the inability to activate Akt due to the presence of F described in Chapter 3 (Figure 3.3) showed F attenuated insulin signalling as early as 2 hours in HepG2 cells; future work could look at the effects shorter F treatment times have on FOXO1 mRNA expression. Both TNF-α and IL-6 showed a slight but not significant increase in FOXO1 mRNA expression, with TNF-α effects appearing more pronounced (Figures 3.7 & 3.8) as seen in Table 4.1. Contrary to this, Miao *et al.* saw no increase in FOXO1 mRNA expression in HepG2 cells pre-treated with TNF-α[375]. Length of exposure to TNF-α could be the reason for this, as Miao *et al.* pre-treated with TNF-α 48 hours before insulin stimulation, compared to Alikhani *et al.*, who saw an increase in FOXO1 expression in fibroblasts treated for 12 hours[373]. To the best of our knowledge, this is the first study that looked at the effect of IL-6 on FOXO1 mRNA expression. As mentioned previously, TNF-α and IL-6 have been implicated in attenuating insulin signalling in nodes upstream of FOXO1, but further investigation on the effects of pro-inflammatory cytokines on FOXO1 is needed. Inflammatory and stress mediators in circulation are not found in insolation and both, as mentioned before, are implicated in insulin resistance and subsequent T2DM. For this reason, it was important to observe the effects of TNF-α and IL-6 on GC-induced FOXO1 mRNA expression (Figures 3.7 & 3.8).

Co-treatment with Dex and TNF-α resulted in an approximately 6-fold increase in FOXO1 mRNA expression, suggesting a co-operative effect on FOXO1 in HepG2. This finding is intriguing as Dex is widely known to be used as an anti-inflammatory treatment[422–425]. Corticosteroid use in diabetic patients are usually avoided, as studies show an increase in hospitalization in diabetic patients given corticosteroids, increased

hyperglycaemia, and additional insulin therapy required[463–465]. Furthermore, healthy individuals given corticosteroids have raised blood glucose, increasing the risk for developing insulin resistance[464,466]. Whether GCs are no longer able to reduce inflammation in insulin resistant patients remains however unclear. F had little effect on FOXO1 mRNA, yet co-treatment with TNF-α caused significant upregulation in FOXO1 mRNA expression, implying that the co-operative effect of TNF-α occurs independently of the type of GC. Interestingly, IL-6 suppressed Dex-induced FOXO1 mRNA expression, despite causing a small increase in FOXO1 mRNA expression alone. The differences observed could be attributed to TNF-α and IL-6 signalling *via* different membrane receptors, which in turn activate different proteins (as discussed in Chapter 1), affecting the GC-mediated effects on FOXO1 differently. Since to the best of our knowledge, this is the first study to observe the effects on FOXO1 by GCs and pro-inflammatory cytokines together, further work needs to be done to elucidate the mechanisms by which TNF-α and IL-6 signalling cross-talk with that of GC signalling.

To elaborate on the findings seen on the mRNA expression of FOXO1, the protein expression was also investigated, as it cannot be assumed that changes in mRNA expression result in similar changes in protein expression. A reliable antibody for FOXO1 could not be obtained and therefore only preliminary results could be displayed (Figure 3.9). Dex at 24 hours appeared to upregulate FOXO1 protein expression (Figure 3.9A), correlating to what was seen at the mRNA level (Figure 3.7) as well as in agreement with other studies in various cell types[174,321,375]. Despite there being no FOXO1 mRNA upregulation induced by F after 24 hours, there did appear to be an increase in F-induced FOXO1 protein expression (Figure 3.9C). It could be that the effects of F on mRNA expression had already diminished to basal levels at the time of cell lysis, but the effects on protein expression remained intact. It is not uncommon for differences between mRNA and protein levels to occur, especially when the same time point is investigated. This is very likely due to various regulatory events that occur between the transcription of mRNA to protein translation[467]. In addition, the rates for synthesis and decay of a specific gene/protein are not related[467]. Nonetheless, the increase in FOXO1 protein expression in response to F is in agreement with a study by Liu *et al.*, which observed an increase in FOXO1 protein expression in mouse hepatoma cells treated with the endogenous GC, Cort[372]. To the

best of our knowledge, the effects of F on FOXO1 protein expression has not yet been observed.

TNF-α resulted in a slight increase in FOXO1 protein expression at 4 hours, consistent with the small increase in FOXO1 mRNA expression induced by TNF-α. Interestingly, IL-6 did not upregulate FOXO1 protein expression at 4 hours despite the slight increase observed in mRNA (Figure 3.7 & 3.8). As mentioned previously, changes in mRNA expression do not always correlate with changes in protein expression. Despite the knowledge that TNF-α and IL-6 are implicated in insulin resistance, no studies have explored their influence on FOXO1 protein expression directly. In fact, several studies describe FOXO1 as being a transcription factor involved in the expression of TNF-α and IL-6, as described in Chapter 1[375,378–380]. Future work would involve confirming the effects these treatments have on FOXO1 protein expression with more biological repeats. In addition, it is important to determine whether the effects of the combination treatments on FOXO1 mRNA expression correlate with FOXO1 protein expression. While the increase in FOXO1 protein expression cannot be confirmed in this study, it is important to note that increased FOXO1 levels only describe how much FOXO1 is present. As described in Chapter 1, FOXO1 can be inactivated by Akt *via* phosphorylation. Thus, to elaborate on this aim, the activation status of FOXO1 was determined and further described in Section 4.5 below.

Table 4.1. Summary of the effect treatment with GCs, cytokines, or a combination thereof have on the mRNA expression of FOXO1 in HepG2 cells (data from Figures 3.7 & 3.8)

Treatment Conditions	Fold-change
Dex	3.5
Dex + TNF-α	5.8
Dex + IL-6	1.3
F	1.1
F + TNF-α	2.5
F + IL-6	0.9
TNF-α	1.7
IL-6	1.3

4.4 Stress indirectly acts on the *foxo1* promoter, while added inflammation produces differential effects

The above aim showed evidence that both GCs and pro-inflammatory mediators influence FOXO1 expression. The way FOXO1 is regulated however, cannot be determined by observing changes in FOXO1 expression alone. Thus, the following aim was to determine whether GCs and pro-inflammatory cytokines had any activity on the *foxo1* promoter. Furthermore, a secondary part of this aim was to investigate whether the activity seen was due to a direct interaction on the promoter or required the assistance of other regulatory proteins. The results in Chapter 3 show that GCs and the pro-inflammatory cytokines do not regulate the transcriptional activity of *foxo1* to a similar extent, and it is likely that unknown interactions outside of promoter activity are involved.

Qin *et al.* showed that the *foxo1* promoter contains several GREs, elements through which GCs *via* the GR are known to activate transcription[174], which could explain why both Dex and F were able to induce activity on the *foxo1* promoter, as seen in Table 4.2. Neither TNF-α, nor IL-6 induced any *foxo1* promoter activity (Table 4.2).

The addition of TNF-α had no effect on either GC-induced *foxo1* promoter activity, although an increase in FOXO1 mRNA expression was observed when HepG2 cells were co-treated with TNF-α and the GCs. In contrast, IL-6 attenuated both Dex- and F-induced *foxo1* promoter activity, in agreement with its effect on Dex-induced increase in FOXO1 mRNA expression. This inhibition by IL-6 possibly involves a downstream regulatory protein such as STAT transcription factors (see Figure 1.7) tethering to the ligand-activated GR inhibiting its transcriptional activity[468]. It is important to note that this study focused on the promoter region of *foxo1* only. It is also possible that the cytokines mediate the transcription of *foxo1* in regions outside of the promoter. This could also explain the slight increase in mRNA detected despite the lack of promoter activity.

To expand our understanding of the molecular mechanism of action whereby GCs and pro-inflammatory cytokines regulate FOXO1, whether *de novo* protein synthesis is required for the regulation of FOXO1 by the test compounds was next investigated. In doing so, it could be determined whether GCs or cytokines influenced the transcription of *foxo1* directly, by comparing changes in mRNA expression by inhibiting the

synthesis of new proteins. CHX was able to inhibit the synthesis of all Dex-induced FOXO1 mRNA expression in Figure 3.13. This implies that Dex initiates the synthesis of an unknown regulatory protein(s) prior to *foxo1* transcription, and that the activity on the *foxo1* promoter occurs *via* this/these protein(s). F had no influence on FOXO1 mRNA expression prior to the addition of CHX, therefore it is not surprising no changes were noted in FOXO1 expression in the presence of both F and CHX. It would however be interesting to determine whether this is simply due to F no longer influencing FOXO1 mRNA at this time point as discussed previously. CHX was unable to significantly reduce TNF-α-induced FOXO1 mRNA expression, despite the decrease observed in Figures 3.13A & 3.14A, which could suggest that TNF-α regulates FOXO1 mRNA expression directly as well as requiring the synthesis of a secondary protein. CHX was unable to significantly inhibit IL-6-induced FOXO1 mRNA expression, despite the decrease observed, indicating IL-6, like TNF-α, regulates FOXO1 mRNA expression directly as well as requires the synthesis of a secondary protein. A summary of the comparison between FOXO1 mRNA induced by these treatments and the addition of CHX is shown in Table 4.2.

The combination treatments shown in Chapter 3 suggest that the regulation of FOXO1 by GCs and TNF-α together requires the synthesis of an unknown regulatory protein. Shorter F treatment times may clarify whether this finding is more significant, due to F having little effect alone at 24 hours. Interestingly, CHX had little effect on Dex and IL-6 treated FOXO1 mRNA, perhaps due to the combination treatment having little effect on FOXO1 mRNA expression, but CHX appeared to result in an increase in F and IL-6 induced FOXO1 mRNA expression (Figure 3.14B). It is possible that CHX prevents IL-6 from activating the synthesis of a regulatory protein involved in inhibiting GCs from eliciting a response. As with previous treatments, additional biological repeats would need to be done to be able to confirm this finding. While CHX has been used in other studies to inhibit protein synthesis, to the best of our knowledge this study is the first to explore effects of GCs and pro-inflammatory mediators on FOXO1 expression in this manner.

The FOXO1 transcription factor plays a role in several mechanisms that vary across tissues; it is therefore not surprising that most research focusses on the transcriptional regulation of genes by FOXO1 rather than the regulation of *foxo1*. Because of this, little is known about the regulation of *foxo1*. Future research could investigate the

promoter of *foxo1* in greater detail, possibly detecting other elements within the promoter, and other regulatory elements outside of the promoter, that may explain changes in the transcription of *foxo1* in insulin resistant states.

Table 4.2. Summary of changes in FOXO1 mRNA expression compared to the effects CHX induced on treatment conditions in HepG2 cells (data from Figures 3.13 & 3.14).

	Fold change in FOXO1 mRNA	Effect of CHX on FOXO1 mRNA	Resulting difference due to CHX
Dex	3.5	1.0	↓
Dex + TNF	5.8	1.5	↓
Dex + IL-6	1.3	1.4	↑
F	1.1	1.0	↓
F + TNF	2.5	1.4	↓
F + IL-6	0.9	1.5	↑
TNF	1.7	1.0	↓
IL-6	1.3	0.8	↓
	Downregulation		Upregulation

4.5 Stress and inflammation inhibit insulin's effect on FOXO1 phosphorylation

Whilst the results of the current study indicates FOXO1 expression in hepatocytes is affected by stress and inflammation-induced insulin resistance, the activation status of FOXO1 cannot be determined from mRNA and protein levels. Thus, to elaborate on these findings, the insulin-induced phosphorylation of FOXO1 was also investigated. As discussed in Chapter 1, the activation of Akt *via* the insulin signalling pathway results in Akt directly phosphorylating FOXO1, inhibiting its transcriptional abilities and subsequently halting gluconeogenesis. Having shown that the GCs at 24 hours and pro-inflammatory mediators at 4 hours inhibit insulin-induced Akt phosphorylation, we wanted to establish whether this attenuation of Akt phosphorylation affects FOXO1 inactivation.

The GCs and pro-inflammatory cytokines do appear to interfere with the insulin-induced phosphorylation of FOXO1 (Figure 3.15), correlating with the

dephosphorylation of Akt, although the extent of inhibition is not as pronounced as what was observed with Akt inactivation. It does however strengthen the observation made that the cells are resistant to insulin due to the test compounds as a downstream target of insulin is affected. To add to this, the GCs appeared to upregulate FOXO1 protein, as discussed in Section 4.3, further compounding the lack of phosphorylated FOXO1 detected. The decrease in FOXO1 phosphorylation observed with the GCs are in agreement with the literature as it has been reported that GCs inhibit the phosphorylation of FOXO1 in the liver[372,375]. Furthermore, several studies have noted an increase in FOXO1 activity in response to TNF-α[375–377], although the effects of IL-6 on FOXO1 activity has not yet been reported. Treatment with either Dex or IL-6, as well as IL-6 in combination with both GCs, appeared to decrease the phosphorylation of FOXO1 to below basal levels. It may be possible that these treatments trigger methylation of FOXO1 which is known to prevent Akt from phosphorylating FOXO1 (see Chapter 1), thereby further reducing FOXO1 phosphorylation[191–194]. Taken together, GCs and pro-inflammatory cytokines appeared to prevent the phosphorylation of FOXO1, indicative of FOXO1 remaining active. This could contribute to PEPCK and G6Pase levels remaining elevated in insulin resistance, contributing to the inability to halt gluconeogenesis and subsequent *de novo* glucose production. In fact, Valenti *et al.* observed a correlation between FOXO1, *g6pc* and *pck1* in the liver of patients with steatohepatitis[469]. In future, more biological repeats are required for this study to determine whether the changes to insulin-induced FOXO1 phosphorylation observed due to GCs and pro-inflammatory cytokines are significant.

4.6 Conclusions and future research

To conclude, both GCs as well as pro-inflammatory cytokines affected insulin signalling, although to different extents in the liver. The pro-inflammatory mediators had shorter acting times on Akt activation, whereas the effects of the GCs could be seen at longer exposure times on Akt. Despite this, all treatments hindered Akt activation. Furthermore, mediators of stress and inflammation were implicated in the expression of FOXO1, a key transcription factor in gluconeogenesis (Table 4.1). Compounding this finding, GCs, and pro-inflammatory mediators, whether alone or treated in combination, inhibited the ability of insulin to phosphorylate FOXO1, which

could have significant effects on the increase in transcription of gluconeogenic enzymes. These results suggest stress and inflammation can establish an insulin resistant state (i.e. attenuation of insulin-induced Akt phosphorylation), as well as cause aberrant FOXO1 regulation, possibly contributing to hyperglycaemia. Thus, our hypothesis that stress and inflammation can cause FOXO1 dysregulation, contributing to the development of insulin resistance can be accepted. To the best of our knowledge, this is the first study to look at the effects of GCs and pro-inflammatory mediators on FOXO1 in the liver to such an extent. This study highlights that the development of insulin resistance by stress and inflammation includes the dysregulation of FOXO1. In addition, it highlights differential effects of the pro-inflammatory cytokines on GC-mediated effects on FOXO1. Interestingly, whilst IL-6 antagonised Dex-mediated increase of FOXO1 mRNA as well as *foxo1* promoter activity, dephosphorylation of FOXO1 by Dex remained unaffected by IL-6. This could be ascribed to IL-6 alone attenuating insulin-induced phosphorylation of FOXO1. It would have been interesting to measure how G6Pase or PEPCK levels are affected by co-treatment with Dex and IL-6 since although mRNA and promoter activity is decreased, the functional pool of FOXO1 i.e., dephosphorylated FOXO1 is increased. Preliminary results from our research group have shown that insulin-mediated inhibition of G6Pase mRNA expression was prevented when HepG2 cells were co-treated with Dex and IL-6 (Addendum D), suggesting that a functional pool of FOXO1 protein is sustained because of the test compounds attenuating insulin-induced FOXO1 phosphorylation. Nonetheless, not having looked at downstream effects of dysregulated FOXO1 activity such as measuring the levels of the key gluconeogenic enzymes as well as glucose synthesis in the hepatocyte cell models is a shortcoming of the current study and should be considered in future studies. In addition, to better understand the molecular mechanism of action, FOXO1 localisation within the cell should be investigated, as functional FOXO1 is found predominately in the nucleus.

Insulin resistance and consequent T2DM development is multi-faceted especially considering the involvement of both inflammation and stress in disease progression. How inflammation and stress mediators contribute to their pathogenesis could possibly also follow different molecular modes of action. However, what this study suggests is that both GCs and pro-inflammatory cytokines affect FOXO1 regulation, both transcriptionally as well as post-translationally. Future studies should explore this to

improve our understanding of the role of FOXO1 in hepatic insulin resistance. This includes determining whether FOXO1 regulation in other insulin sensitive tissues are also affected considering gluconeogenesis also occurs in muscle tissue. Determining the mechanism in which pro-inflammatory cytokines and GCs exhibit their effects on the regulation of *foxo1* could provide insight into how stress and inflammation cause dysregulation in gluconeogenesis. In addition, it would be interesting to determine to what extent FOXO1 contributes to stress and inflammation-induced insulin resistance.

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Addendum A: Additional information

A1. All cell lines were tested for mycoplasma and found to be negative.

Mycoplasmas are small, prokaryotic bacteria that lack a cell wall. In cell culture, mycoplasma contamination can alter the physiology of cells, thus resulting in results that may not accurately reflect what is seen *in vitro*[470]. Mycoplasma cannot be seen by the naked eye and can often remain undetected without noticeable changes to cells. For this reason, testing for mycoplasma regularly is important to ensure accuracy and reliability of results.
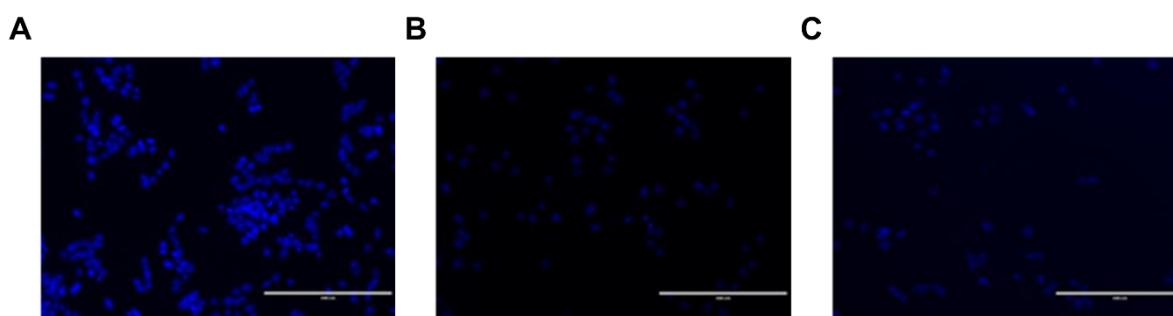
Figure A1. Mycoplasma negative BWTG3 (A), COS-1 (B), and HepG2 (C), were fixed using methanol and glacial acetic acid (3:1 ratio) before Hoechst 33258 DNA staining (Sigma-Aldrich). Fluorescent images were obtained using the EVOS imaging system (Thermo Fisher).

A2. RNA isolated from HepG2 cells were confirmed to be intact.

Observing gene expression is an important component in many studies, with qPCR being a widely used technique to do this. In order to synthesize cDNA to be used in qPCR experiments, RNA is required as a template. The quality and purity of RNA extracted from cell culture is therefore essential, as poor-quality RNA can affect the accuracy of experimental results[471]. Thus, determining the quality and assessing the integrity of RNA prior to cDNA synthesis was performed for each biological repeat in this study, as depicted by the representative agarose gel in Figure A2. The integrity of RNA is considered of high quality when both 28S and 18S rRNA can be identified as sharp, clear bands on an agarose gel, and the 28S band usually appears more intense compared to the 18S band. Furthermore, no other bands should be present on an agarose gel to ensure the purity of the RNA samples.
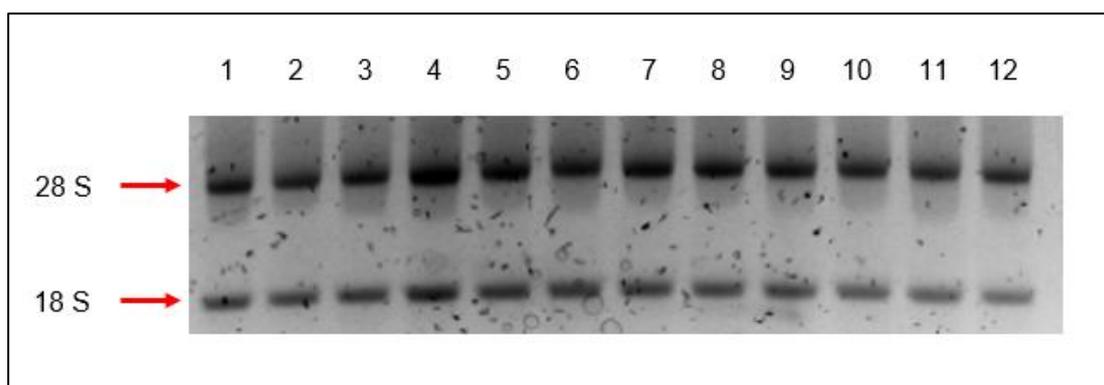
Figure A2. Representative 1% agarose gel showing intact RNA isolated from HepG2 cells. RNA was isolated from HepG2 cells using the method described in Chapter 2. To determine RNA integrity for cDNA synthesis, 1µg RNA was loaded onto an agarose gel and visualized with Nancy-520 nucleic acid stain on the Bio Rad ChemiDoc XRS imaging system. Lanes 1 - 2 (vehicle control), lanes 3 – 4 (insulin control), lane 5 (Dex), lane 6 (Dex + TNF-α), lane 7 (Dex + IL-6), lane 8 (F), lane 9 (F + TNF-α), lane 10 (F + IL-6), lane 11 (TNF-α), lane 12 (IL-6).

A3. FOXO1 primers were optimized and the FOXO1 product size confirmed.

The qPCR technique is considered a sensitive, and precise method for the measurement and quantification of RNA or DNA. qPCR requires several components of which primers are responsible for the specific amplification of the desired product. For this reason, it is important to optimize the primers required to ensure the specificity of the primer pair and eliminate the possibility of off-target amplification or primer dimers. Thus, a temperature gradient was performed to determine the optimal melting temperature at which a single product was obtained (Figure A3.A), and this product was loaded onto an agarose gel to confirm that the size of the product correlated with what was expected for FOXO1 (Figure A3.B). The optimal annealing temperature for the human FOXO1 primers selected was 63°C (lane 9 in Figure A3.A), as this temperature resulted in a single band of high intensity on an agarose gel, indicating the amplification of a single product confirmed to be of the correct bp length.

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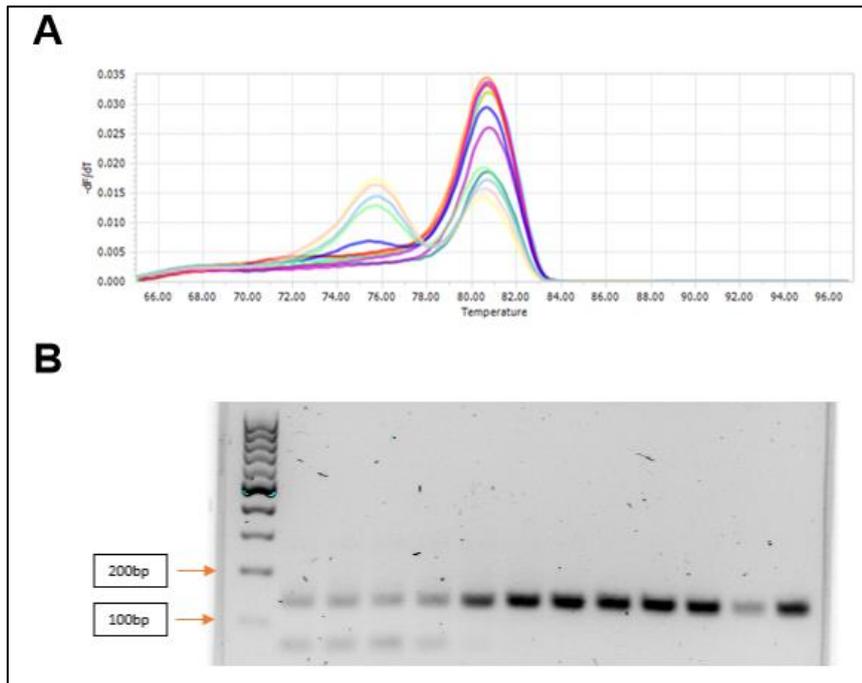
Figure A3. Temperature gradient melting peaks and qPCR product gel confirmed the optimal temperature and confirms amplification of FOXO1 mRNA. Human FOXO1 primers were optimized by determining the optimal annealing temperature **(A)** and confirming the qPCR product on a 2% (w/v) agarose gel **(B)** visualized with Nancy-520 nucleic acid stain. The optimal annealing temperature selected was 63°C (lane 9).

A4. Confirming the size and integrity of plasmids used for promoter-reporter assays.

The use of plasmids in promoter reporter assays provides the ability to insert a gene of interest into cells that can then be detected to observe changes in gene expression. Prior to transection, it is important to assess whether the plasmid is of the correct size, as well as the integrity of the plasmid. For this reason, an enzyme was selected that performed a single cut in the vector to linearize the plasmid, thereby confirming the size of the plasmid, and an undigested sample was loaded onto an agarose gel to determine whether the plasmid's integrity was supercoiled, as this is required for optimal transfection (Figure A4).
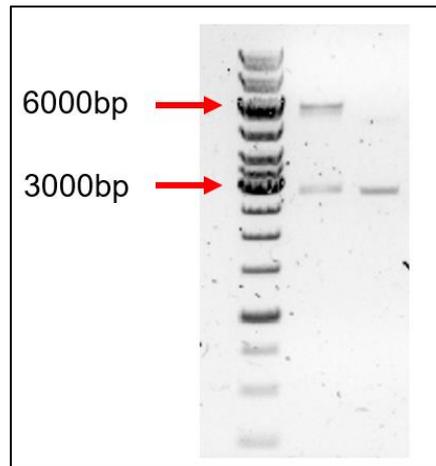
Figure A4. Confirming size and integrity of pGL3-mFOXO1wt-luc. The size and the integrity of the pGL3-mFOXO1wt-luc FOXO1 plasmid was confirmed using a restriction enzyme digest. The plasmid was digested by the Xho I enzyme; both digested (lane 2) and the undigested plasmid (lane 3) loaded onto a 0.5% (w/v) agarose gel and visualized with Nancy-520 nucleic acid stain. Plasmid size is 6056bp in size. Marker: O' GeneRuler 1kB.

Addendum B: Confocal microscopy optimization

Investigating ligand-induced effects on FOXO1 translocation

It is well known that in the presence of insulin, FOXO1 is phosphorylated and moves from the nucleus to the cytoplasm, thereby ending the proteins' ability to transcribe gluconeogenic genes, as discussed in Section 1.3. Since the phosphorylation of FOXO1 was affected by both GCs and pro-inflammatory cytokines (Chapter 3), it was therefore of interest to determine whether the translocation is also affected in the presence of GCs and pro-inflammatory cytokines. To explore this further, the location and movement of GFP-tagged FOXO1 upon the addition of insulin was to be determined using confocal microscopy. Furthermore, the effects stress and inflammation have on the insulin-induced translocation of the FOXO1 transcription factor was to be investigated.

Transfection of cell lines

Cell lines that were used in this experiment included a human hepatoma derived cell line (HepG2) and a monkey kidney epithelial cell line (COS-1). The COS-1 cells purchased from American Type Culture Collection (USA), whilst the HepG2 cells were purchased from Cellonex.

This experiment made use of a GFP-tagged FOXO1 insert in a pEGFP-N1 vector, named pEGFP-N1 Foxo1. This plasmid was purchased from AddGene. Furthermore, COS-1 cells were co-transfected with pEGFP-N1 Foxo1 and an untagged GRα plasmid, pRS-GRα, a kind gift from Ronald M. Evans, the Salk Institute (San Diego, California).

Method 1:

Cells were plated into 6-well plates at a density of 2×10^5 cells/well containing a sterile coverslip. 24 hours after seeding and cells have reached 60-70% confluency, pEGFP-N1 Foxo1 plasmid was transiently transfected using X-tremeGENE 9 transection reagent (1µg DNA to 2µl X-tremeGENE, or 1µg DNA to 4µl X-tremeGENE) as described by the manufacturer (Roche, Germany). COS-1 cells were co-transfected with pEGFP-N1 Foxo1 and pRS-hGRα. During experiments, cells were transfected in DMEM supplemented with 1% Pen/Strep and 10% FCS and left for 24 hours at 37°C,

95% humidity and 5% CO_2. Cells were rinsed once with cold 1X phosphate buffered saline (PBS) before 100ng/ml insulin was added in un-supplemented DMEM for either 15 minutes or 30 minutes before cell lysis.

Method 2:

Cells were plated as described in method 1. After seeding for 24 hours, the supplemented medium was removed and replaced with a mixture containing 5ml supplemented DMEM, 50µl Diethylaminoethyl (DEAE), 5µl chloroquine antibiotic, and the appropriate volume for selected concentration of plasmid DNA. Cells were incubated for 4 hours at 37°C, 95% humidity and 5% CO_2. Following this, the mixture was removed, and Dimethyl Sulfoxide (DMSO) added briefly to initiate DNA uptake. Cells were rinse in 1X PBS before adding supplemented DMEM and incubated for another 24 hours. Cells were replated and treated with insulin as described in method 1.

Preparation of microscopy slides

Method 1:

Plates were removed from the incubator and rinsed with methanol: glacial acetic acid in a ratio of 3:1. Following this, the same methanol: glacial acetic acid mixture containing DRAQ5® (Thermo Fisher) was added for 5 minutes at 37°C to stain the nucleus. Cover slips were gently removed from wells of the 6-well and mounted to microscope slides using Dako fluorescent mounting medium.

Method 2:

After plates were removed from incubation, 4% formaldehyde was added to each well along with 10nM DRAQ5® for 15 minutes covered in foil. Coverslips were removed and mounted to microscope slides as in method 1.

Preliminary results and discussion

Firstly, the plasmid concentration had to be optimized in HepG2 cells. Accili *et al.*, who synthesized the GFP-tagged FOXO1 plasmid, treated with 2.5µg/ml DNA[472]. Initially, HepG2 cells were transfected with GFP-tagged FOXO1 in a 10cm^2 dish at a concentration of either 2µg/ml or 1µg/ml and left for 24 hours before re-plating into 6-wells, and subsequently left for an additional 24 hours before treating. This resulted in

very few cells becoming transfected, if any (see Figure B1.A & B). This could have been because the concentrations selected were lower than what was used by Accili *et al*. Furthermore, transient transfections can only be used to explore short-term protein expression, as plasmid DNA is not integrated into the cells' genome and is lost during cell division[473]. It was of concern that this was occurring, due to the absence of a signal. Thus, it was decided that for following optimization steps, cells would be directly plated into 6-wells with coverslips and transfected in the wells directly. It was decided to continue using a concentration of 2µg/ml but change the transfection reagent ratio from 1µg DNA to 4µl transfection reagent (1:4), to a 1:2, as the optimal ratio is often cell dependent. This resulted in an increase in cells transfected, however, this was inconsistent and too few cells in each repeat to quantify. Furthermore, there was high background signal detected that made it difficult to distinguish the location of GFP-tagged FOXO1 within cells (see Figure B1.C). The HepG2 cell line has a tendency to stack when grown in cell culture, making it difficult to transfect due to reduced surface area. For this reason, it was decided to use the COS-1 for further microscopy experiments. Unfortunately, the COS-1 cell line lacks endogenous GR, which would inhibit any GC-mediated effects. Thus, COS-1 had to be optimized for co-transfection with both pEGFP-N1 Foxo1 and pRS-GRα plasmids. It was decided to select 5µg GFP-tagged FOXO1 and 500ng GR plasmid in the COS-1 cell line, with a DNA to transfection reagent of 1:2 (see Figure B1.D). Despite the low transfection efficiency, the treatment time for insulin action needed to be optimized. Insulin was added for 10 minutes or 30 minutes to observe movement of GFP-tagged FOXO1 from the nucleus. It appeared that 10 minutes was enough time for FOXO1 to move out of the nucleus, although it should be noted that this was not consistently seen since some repeats remained untransfected (Figure B1. E & F). Following this, the method used to fix the cover slips was changed, since it was thought that perhaps the methanol: acetic acid diminished the signal[473]. There was, however, little change in signal observed using the 4% formaldehyde over the methanol: acetic acid fixing method. Subsequently, it was decided to investigate other transfection methods to see whether transfection efficiency could be increased. The DEAE transfection method was selected, however this could not be taken further due to time constraints.

In order to determine whether stress- and inflammation-induced insulin resistance alters nuclear translocation of FOXO1, further experiments need to be conducted to

optimize the transfection of cell lines in order to get a clear signal that can be quantified. Furthermore, the results obtained from confocal microscopy can be supplemented using nuclear fractionation experiments.
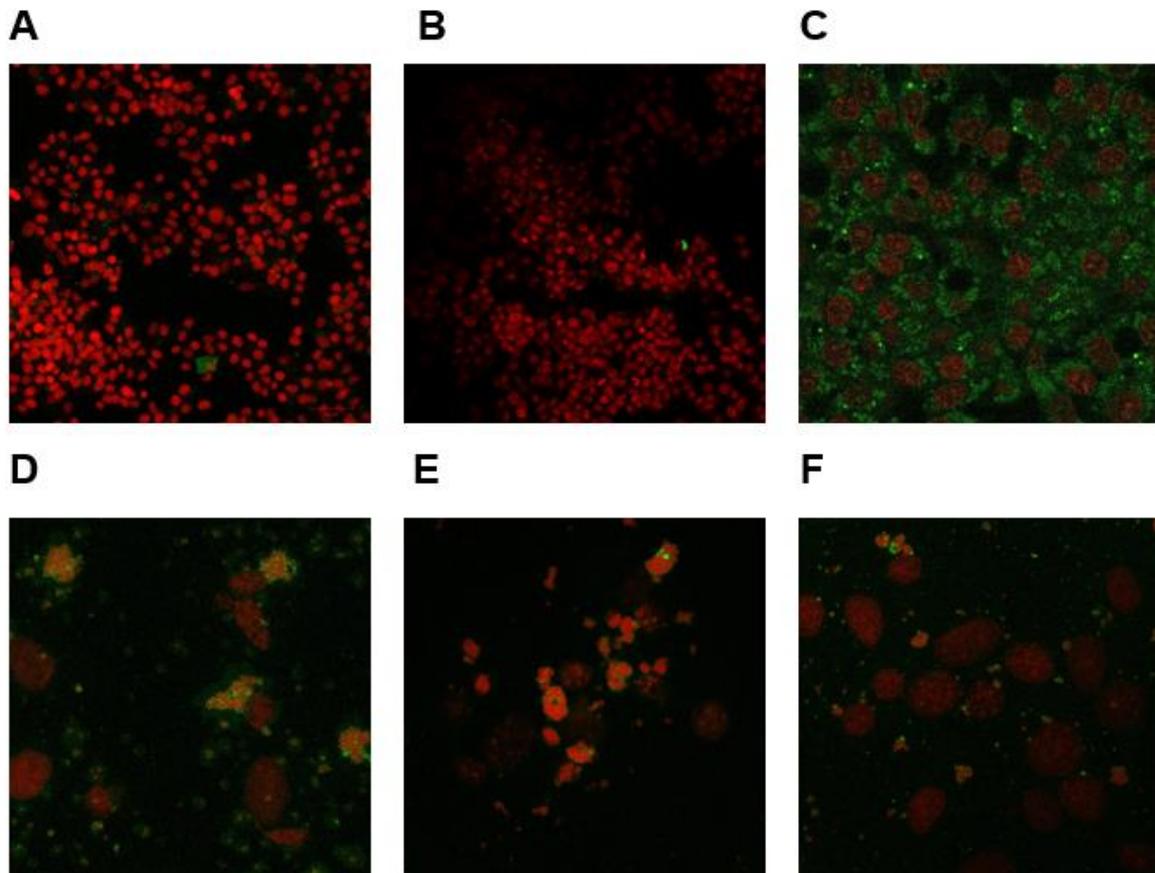

Figure B1. Optimizing the transfection of GFP-tagged FOXO1 in two cell lines, HepG2 and COS-1, determined using confocal microscopy. The HepG2 cell line **(A – B)** had great difficulty taking up the pEGFP-N1 Foxo1 plasmid. Only when the light exposure was increased **(C)** was fluorescence present, but the majority of this appeared to be background signal detected. In the COS-1 cells **(D – F)** the cells also show very little fluorescence. COS-1 shown here without insulin **(D)**, with 10 minutes of insulin exposure **(E)**, and with 30 minutes of insulin exposure **(F)**.

Addendum C: Loading controls used for western blotting

The western blot is often used to compare a protein of interest amongst samples that differ in treatment conditions, disease states, or other biological variables. In order to accurately measure the protein levels across selected samples, a loading control is required. A loading control confirms whether any changes seen between the protein of interest in selected samples are representative of actual differences observed. Generally, a loading control is a protein whose presence across samples remains unaffected by experimental conditions. Loading controls can be used to adjust for variability that is present between samples in a process referred to as normalization. Normalization improves the accuracy and reproducibility of western blot analysis. In order for a loading control protein to be used for normalization of a protein of interest in western blotting, it needs to be known that the loading control is not affected by selected treatment conditions. For this reason, the loading control proteins Hsp90 (Figure C1 & C3) and EF1-α1 (Figure C2) used in this study were compared across treatment conditions to show that these proteins remain unaffected and are therefore suitable to be used for normalization of a protein of interest.
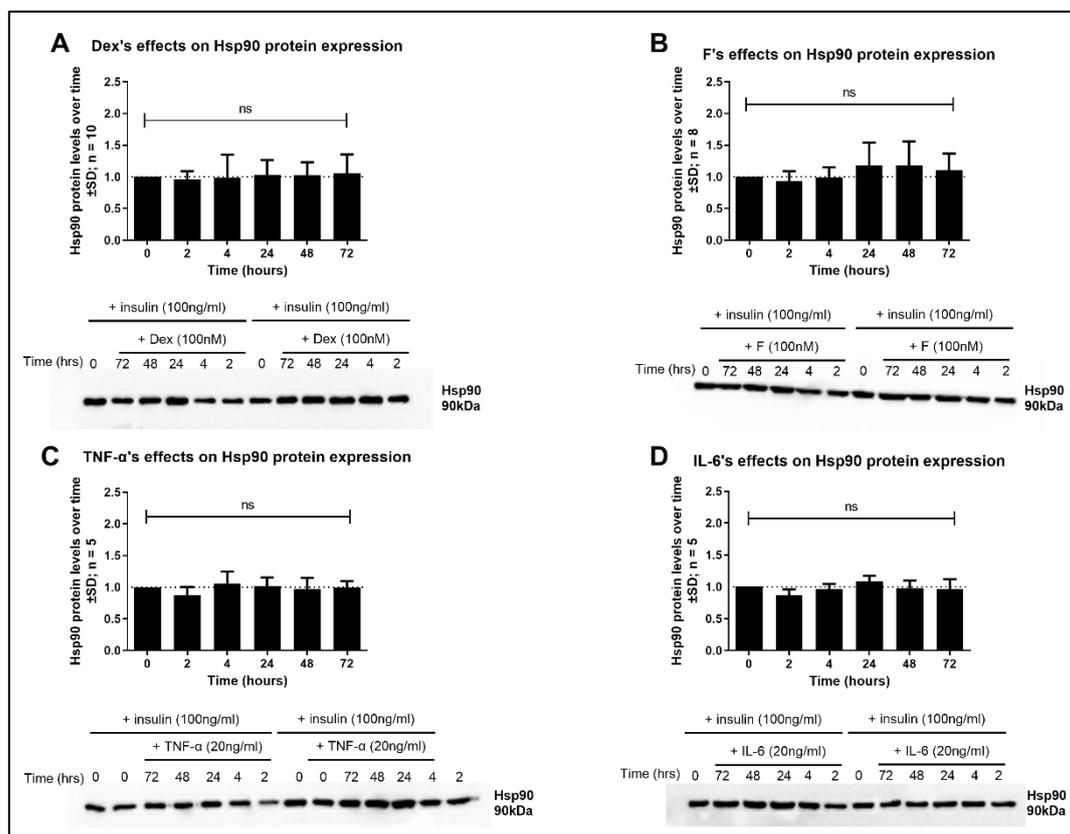
(Figure legend on next page).

Figure C1. The protein Hsp90 is unaffected by GCs and pro-inflammatory cytokines used in this study. HepG2 cells were cultured and maintained in low glucose medium. After serum starving for 24hrs, cells were treated with 100nM Dex **(A)**, 100nM F **(B)**, 20ng/ml TNF-α **(C)**, or 20ng/ml IL-6 **(D)** for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. The Hsp90 levels were compared to a vehicle control (set to 1) represented by the dotted line. Statistical analysis comparing Hsp90 under these treatment conditions was performed using one-way ANOVA statistical analysis with Dunnett's post-test and found none of the different time points to be significantly different from 0 hours, represented by 'ns' for each test compound.


Figure C2. The protein EF1-α1 is not regulated by GCs and pro-inflammatory cytokines used in this study. HepG2 cells were cultured and maintained in low glucose medium. After serum starving for 24hrs, cells were treated with 100nM Dex **(A)**, 100nM F **(B)**, 20ng/ml TNF-α **(C)**, or 20ng/ml IL-6 **(D)** for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. The EF1-α1 levels were compared to a vehicle control (set to 1) represented by the dotted line. Statistical analysis comparing EF1-α1 under these treatment conditions was performed using one-way ANOVA statistical analysis with Dunnett's post-test and found none of the different time points to be significantly different from 0 hours, represented by 'ns' for each test compound.
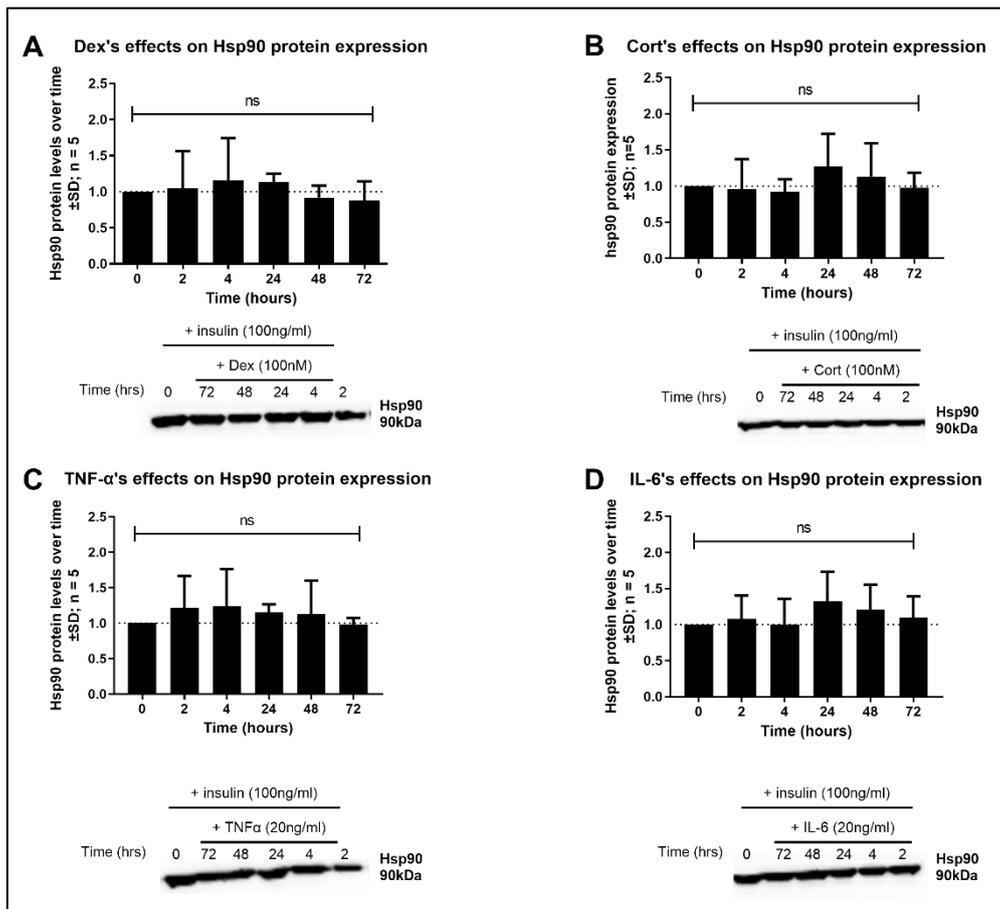
Figure C3. The protein Hsp90 is unaffected by GCs and pro-inflammatory cytokines used in this study. BWTG3 cells were cultured and maintained in low glucose medium. After serum starving for 24hrs, cells were treated with 100nM Dex **(A)**, 100nM Cort **(B)**, 20ng/ml TNF-α **(C)**, or 20ng/ml IL-6 **(D)** for either 2, 4, 24, 48, or 72hrs before cell lysis. 100ng/ml insulin was added 30min prior to cell lysis. The Hsp90 levels were compared to a vehicle control (set to 1) represented by the dotted line. Statistical analysis comparing Hsp90 under these treatment conditions was performed using Statistical analysis comparing Hsp90 under these treatment conditions was performed using one-way ANOVA statistical analysis with Dunnett's post-test and found none of the different time points to be significantly different from 0 hours, represented by 'ns', for each test compound.

Addendum D: Preliminary G6Pase mRNA results obtained by N Green

Preliminary data obtained by Nicole Green in her MSc thesis showing the effects of Dex in the absence and presence of TNF-α or IL-6 on insulin-induced inhibition of G6Pase mRNA expression. G6Pase is a key gluconeogenic enzyme regulated by the transcription factor FOXO1.
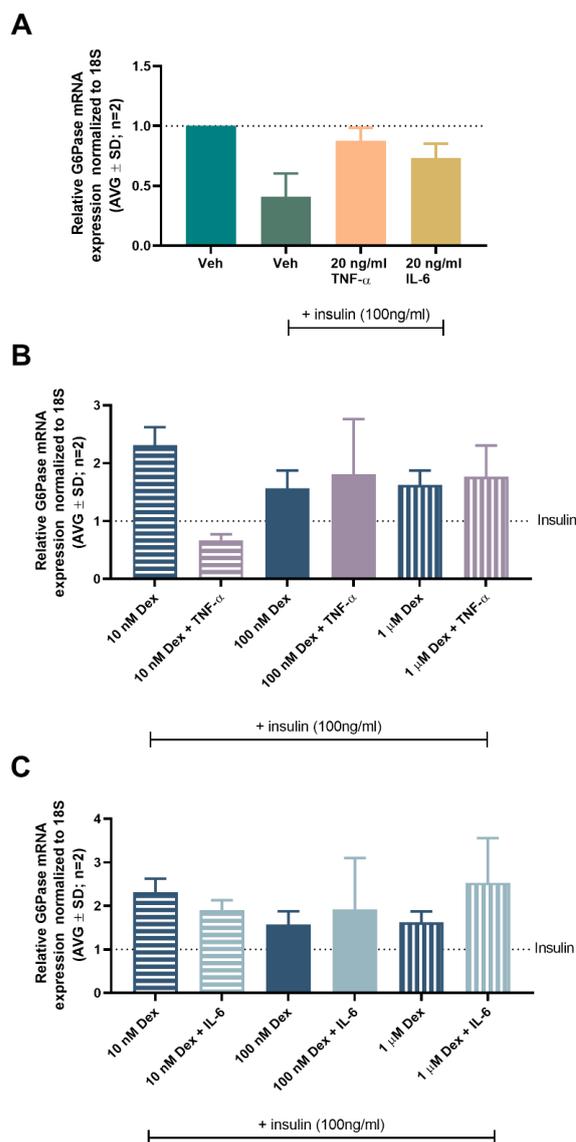

Figure D1. Preliminary data showing the insulin-mediated decrease of G6Pase mRNA is affected by Dex and the pro-inflammatory cytokines, TNF-α and IL-6. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μM Dex, or 20 ng/ml of either TNF-α or IL-6. For co-treatments, cells were treated with a combination of Dex at the various concentrations and either of the two cytokines. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin for 30 minutes before lysis to stimulate the insulin signalling pathway. G6Pase mRNA expression was measured and normalised using 18S as the housekeeping gene. In (**A**) vehicle control was set to 1, whereas in (**B**) and (**C**) the response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control, to determine how the test compounds affect insulin. Images are separated by cytokine type, i.e., TNF-α **(B)** and IL-6 **(C)**. Data shown represents two independent experiments. (*Data of Nicole Green; MSc Thesis*)