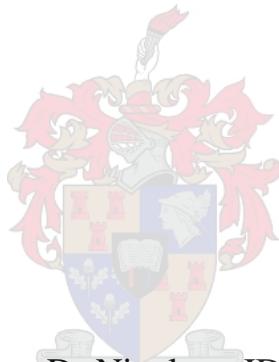


Co-regulation of insulin signalling by glucocorticoids and pro-inflammatory cytokines

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ABSTRACT

In the liver, insulin is responsible for maintaining glucose homeostasis by activating glycogen synthesis and inhibiting *de novo* glucose synthesis. Interruption or improper functioning of the insulin signalling pathway is one mechanism by which insulin resistance (inadequate response of target tissues to normal insulin levels) occurs. Prolonged exposure to glucocorticoids (GCs) has been linked to increased hepatic glucose production, and subsequently insulin resistance. Furthermore, chronic inflammation (represented by pro-inflammatory cytokines) has been implicated in the interruption of insulin signalling, also leading to insulin resistance. However, these two biological mediators, which are seldom present in isolation, serve opposing functions in the body with GCs having anti-inflammatory action. The fact that chronic stress and chronic inflammation result in the same consequence in the form of insulin resistance led us to question whether these biological mediators are able to co-regulate insulin signalling at key insulin regulated nodes namely, AKT- and GSK-3 protein expression and phosphorylation as well as the G6Pase mRNA expression in both murine (BWTG3) and human (HepG2) hepatoma cell lines. Overall, co-regulation by the GCs and cytokines was observed at all nodes except GSK-3 α . The results, however, were GC, cytokine, and cell line specific. As expected, antagonistic behaviour was displayed between the GCs and cytokines, but interestingly, co-operative effects were also observed. Whether these biological mediators lead to an insulin resistant state when present simultaneously *in vivo*, remains to be determined.

OPSOMMING

In die lewer is insulien verantwoordelik vir die handhawing van glukose-homeostase deur glikogeen-sintese te aktiveer en *de novo*-glukose-sintese te inhibeer. Onderbreking of onbehoorlike werking van die insulienseinweg is een meganisme waardeur insulienweerstandigheid (onvoldoende reaksie van die teikenweefsel op normale insulienvlakte) plaasvind. Langdurige blootstelling aan glukokortikoïede (GC's) is gekoppel aan verhoogde lewerglukoseproduksie, en gevvolglik insulienweerstandigheid. Voorts word daar gedink dat chroniese inflammasie (verteenwoordig deur pro-inflammatoriese sitokiene) die onderbreking van die insuliensein tot gevolg het, wat ook tot insulienweerstandigheid lei. Hierdie twee biologiese seintransduksiemolekules, wat selde in isolasie teenwoordig is, dien egter teenoorgestelde funksies in die liggaam, met GC's wat 'n anti-inflammatoriese werking het. Die feit dat chroniese spanning en chroniese inflammasie dieselfde gevolg het, in die vorm van insulienweerstandigheid, het ons laat twyfel of hierdie biologiese molekules die vermoë het om mede-reguleerders te kan wees van insulienseinsending by sleutel-insuliengereguleerde nodusse, naamlik AKT- en GSK-3 proteïen-uitdrukking en fosforilering sowel as die G6Pase mRNA-uitdrukking in beide muriene (BWTG3) en menslike (HepG2) hepatoom-sellyne. In die algemeen is mede-regulering deur die GC's en sitokiene by alle nodusse waargeneem, behalwe GSK-3 α . Die resultate was egter GC-, sitokien- en sellyn-spesifiek. Soos verwag is antagonistiese gedrag tussen die GC's en sitokiene vertoon, maar interessant genoeg is samewerkingseffekte ook waargeneem. Of hierdie biologiese molekules tot 'n insulienweerstandige toestand lei as dit gelyktydig *in vivo* voorkom, moet nog bepaal word.

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ALPHABETICAL LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
bp	Base pair
cDNA	Complementary DNA
CRP	C-Reactive protein
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
EF-1 α 1	Elongation factor-1 alpha 1
FBS	Foetal bovine serum
FoxO1	Forkhead box protein O1
G-6-P	Glucose 6-phosphatase
G6Pase	Glucose 6-phosphatase
GC	Glucocorticoid
GLUT	Glucose transporter
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3
HGP	Hepatic glucose production
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
IKK β	Inhibitor of nuclear factor kappa B subunit beta
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IR	Insulin receptor

IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NF κ B	Nuclear factor kappa B
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP ₂	Phosphatidylinositol (4,5)-bisphosphate
PIP ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PTB	Phosphotyrosine binding
PP-1	Protein phosphatase 1
p-AKT	Phosphorylated AKT
p-GSK-3	Phosphorylated GSK-3
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SH2	Src-homology 2
T2DM	Type 2 diabetes Mellitus
TBS	Tris-buffered saline
TNF- α	Tumour necrosis factor-alpha

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CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

The International Diabetes Federation reported that around 90% of all diabetes cases is type 2 diabetes mellitus (T2DM), affecting millions of people worldwide, including in South Africa (1–4). The physiological features of T2DM include obesity, hypertension, atherosclerosis, and dyslipidaemia (5) to name but a few. Insulin resistance, which is described as the inability of cells to effectively respond to normal insulin concentrations (6), is one of the proposed mechanisms that links all of these features and is a major characteristic and predictor of T2DM (7–9). In the case of T2DM, insulin resistance goes hand-in-hand with insufficient insulin secretion in response to the demand for higher concentrations of insulin needed (1).

The insulin signalling pathway plays a crucial role in the development of insulin resistance as changes to the expression and/or phosphorylation state of any of the key nodes in the signal transduction pathway of insulin could lead to insulin resistance (6, 10–15). A number of factors have been documented to lead to the development of insulin resistance and subsequently T2DM, including increased exposure to glucocorticoids (GCs), whether endogenous or exogenous (9, 16, 17), and chronic inflammation (9, 18–21).

GCs for example, have been shown to cause a decrease in insulin sensitivity in insulin target tissues (22). In the liver, increased concentrations of GCs results in increased hepatic glucose production as a consequence of increased gluconeogenesis and glycogen breakdown (15, 21, 23, 24). Decreased insulin sensitivity in adipose tissue due to over-exposure to GCs, results in decreased glucose uptake as well as increased lipolysis (25, 26). The inability of skeletal muscle cells to effectively take up glucose, is a consequence of reduced insulin sensitivity by GCs (27, 28). Moreover, a decrease in glycogen synthesis as well as an increase in proteolysis in skeletal muscle cells occurs (29). All of these lead to the development of hyperglycaemia and subsequent development of T2DM (6, 28, 30).

Similar to GCs, chronic inflammation is associated with the development of insulin resistance (8, 31–33). A known mechanism by which inflammation causes insulin resistance in insulin target tissues is by interfering with insulin signal transduction (8, 31, 32, 34). Chronic expression of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) have been linked to the suppression of insulin signalling (8, 20, 34–36). These inflammatory mediators have been proposed to target multiple nodes in the insulin signalling pathway thereby negatively affecting any downstream insulin signalling and resulting in impaired glucose metabolism (37, 38). Inflammatory mediators such as pro-

inflammatory cytokines, through negatively influencing insulin signal transduction have been implicated in inducing gluconeogenesis and inhibiting glycogen synthesis (33, 39–41).

GCs and pro-inflammatory cytokines are well-known for their opposing physiological roles, with GCs being used as an anti-inflammatory treatment for decades (42, 43). Thus, it begs the question, why/how, despite having antagonistic functions, do the increased exposure to GCs and pro-inflammatory cytokines both result in insulin resistance? There is no data available, to the best of our knowledge, regarding any co-regulation of insulin signalling by GCs and pro-inflammatory cytokines. As mentioned earlier, insulin signalling comprises various nodes that require careful regulation, which could be targets for GCs and pro-inflammatory cytokines. The protein levels of key proteins such as AKT (also known as protein kinase B) can be regulated, affecting any downstream signalling (44, 45). In addition, the activity of this central protein is regulated by its phosphorylation status at specific amino acid residues. Full activation of AKT for example is achieved when it is phosphorylated at serine 473 and threonine 308, which is required for downstream signal amplification and the subsequent metabolic effects of insulin (44, 46, 47). Immediately downstream from AKT is the enzyme glycogen synthase kinase-3 (GSK-3), the protein levels and activation status of which can also be regulated and possibly targeted (48, 49). Insulin signalling *via* AKT is responsible for the inactivation of GSK-3 through phosphorylation of this downstream protein at specific serine residues thereby inactivating its kinase activity and thus preventing the inactivation of glycogen synthase (GS) (50). Furthermore, GSK-3 requires phosphorylation at distinct tyrosine residues to be considered fully active as a kinase and thereby inactivating GS through phosphorylation and consequently inhibiting glycogen synthesis (50, 51). Additionally, in the liver for example, insulin signalling leads to the inhibition of gluconeogenesis by preventing the transcription of key gluconeogenic enzymes such as glucose-6-phosphatase (G6Pase) through the inhibition of transcription factors responsible for their gene regulation (52, 53). This also may be considered a node of the pathway which can be regulated and possibly targeted by insulin resistance causative agents.

This review of the literature will focus on how GCs and pro-inflammatory cytokines disrupt normal insulin signalling, identifying similar key regulatory targets and convergence in their respective signal transduction pathways. But firstly, the insulin signalling pathway will be discussed to provide background to unperturbed insulin signalling followed by the molecular events involved in establishing insulin resistance.

1.2 Function of insulin in insulin target tissues

Insulin is an anabolic, peptide hormone (6, 54) and it affects a host of physiological processes, such as carbohydrate and lipid metabolism (55). However, it is best known for regulating glucose homeostasis (56). The primary driver of insulin secretion is glucose. An increase in blood glucose concentration induces glucose-mediated insulin secretion from secretory granules in the β -cells of the pancreas (6). Insulin has both metabolic and mitogenic effects in the body. It is responsible for maintaining normal glucose levels in the blood (metabolic effects) as well as cell division and growth (mitogenic effects) (6).

Insulin has three main target tissues in the body namely the liver, skeletal muscle, and adipose tissue. The primary focus of this review will be the effects of insulin in the liver.

1.2.1 Function of insulin in the liver

The liver plays a vital role in energy homeostasis, with one of its most essential functions being the regulation of glucose input and output, otherwise known as glucose metabolism (57). In humans, insulin is secreted into the portal venous system, where 40-80% of this insulin is then cleared by the liver. Therefore, the liver is always exposed to higher insulin concentrations than other organs (58–60). The main function of insulin in the liver is to inhibit glucose production. After a meal, an increase in insulin concentration signals the liver to 1) take up glucose, 2) to store this glucose as glycogen, and 3) to halt the *de novo* synthesis of glucose via gluconeogenesis by repressing the mRNA synthesis of key gluconeogenic enzymes such as G6Pase and phosphoenolpyruvate carboxykinase (PEPCK) (61, 62).

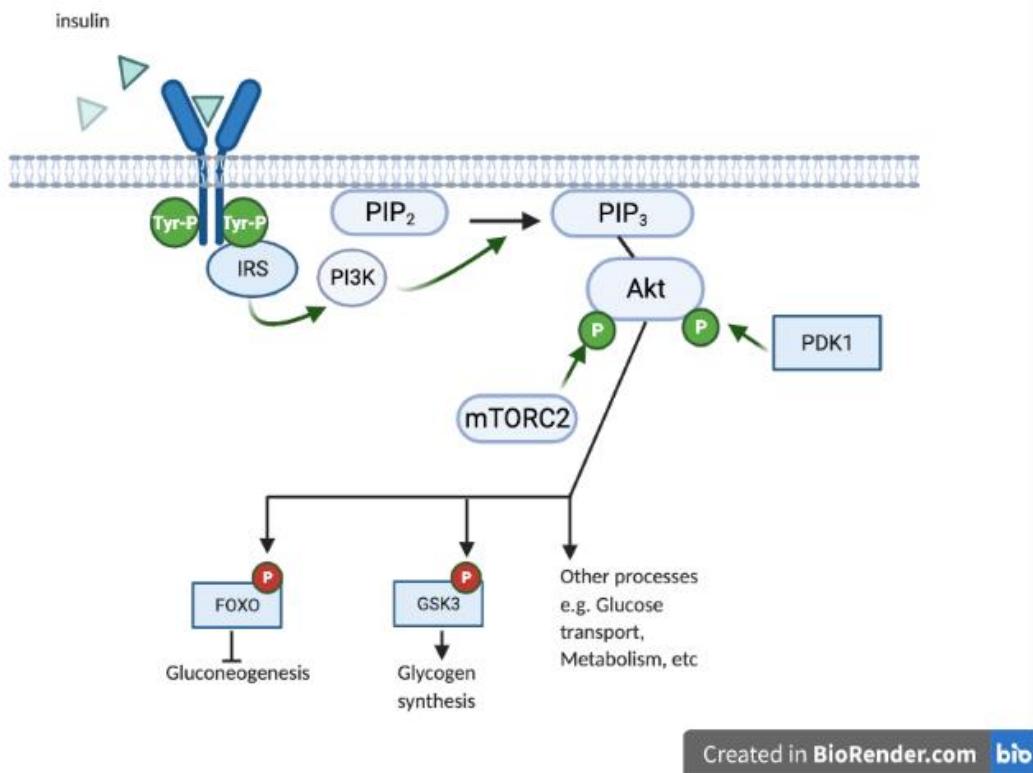
1.2.2 Function of insulin in skeletal muscle and adipose tissue

The main effects of insulin in the muscle and adipose tissue involve carbohydrate, lipid, and protein metabolism. These include increasing the rate of glucose transport across the cell membrane as well as increasing the rate of glycolysis (29, 63). Insulin's effect on skeletal muscle cells, as in the liver cells, is responsible for an increase in glycogen synthesis (63). In response to insulin, adipocytes are responsible for the storage of nutrients in the form of triacylglycerol (TAG), a compact and efficient form of carbohydrate storage that is readily available to be mobilised in time of need, such as during fasting (47, 63). Insulin also inhibits protein synthesis in skeletal muscle and inhibits lipolysis in adipocytes, hereby reducing the amount of free fatty acids (FFAs), glycerol and amino acids available to be used as substrates for gluconeogenesis (64–67).

1.3 Insulin signalling

The effects of insulin on its target tissues mentioned above involves an intricate and complex signalling cascade with many points of regulation, some of which were briefly mentioned in the introduction (section 1.1) and depicted in Figure 1.1 (8, 68). The binding of insulin to the insulin receptor (IR), an integral membrane protein belonging to the receptor tyrosine kinase family, leads to the activation of two key pathways: the phosphoinositide 3-kinase (PI3K)-AKT pathway, which is the pathway responsible for the metabolic effects of insulin, and the Ras-MAPK (mitogen-activated protein kinase) pathway, responsible for the mitogenic effects of insulin (68). For the purpose of this thesis, we will focus mainly on the PI3K-AKT pathway.

Upon reaching insulin-responsive tissues, insulin binds the extracellular, α -subunit of the dimeric IR. This then consequently leads to the autophosphorylation of the intracellular, β -subunit of the receptor at specific tyrosine residues (69). Adaptor proteins known as insulin receptor substrates (IRS) are then recruited to the IR, binding to the plasma membrane *via* their pleckstrin homology (PH) domain, which recognises phosphatidylinositol present in the plasma membrane, as well as binding to the phosphorylated tyrosine residues of the IR *via* their phosphotyrosine binding (PTB) domains (30). Binding of the IRS adaptor proteins to the ligand-activated IR leads to its own phosphorylation, also at tyrosine residues, thus creating binding sites for intracellular proteins containing Src-homology 2 (SH2) domains. IRS essentially acts as a protein scaffold linking the membrane-bound ligand-activated receptor to intracellular signalling proteins (30, 69). The tyrosine phosphorylated IRS molecules allow for the recruitment of PI3K to the membrane and PI3K in turn becomes activated. Activated PI3K consequently phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP₂) anchored to the cell membrane to generate phosphatidylinositol (3,4,5)-triphosphate (PIP₃). PIP₃ will activate phosphoinositide-dependent kinase 1 (PDK1), which is responsible for the phosphorylation of AKT (also referred to as PKB and considered the central protein in the pathway) at its threonine 308 residue leading to its activation. Additionally, AKT gets phosphorylated at a second site, serine 473, by mammalian target of rapamycin (mTOR) complex 2. Both phosphorylation events are required for the full activation of AKT, allowing AKT to signal phosphorylation control of many downstream metabolic processes, such as gluconeogenesis (44, 46, 47).

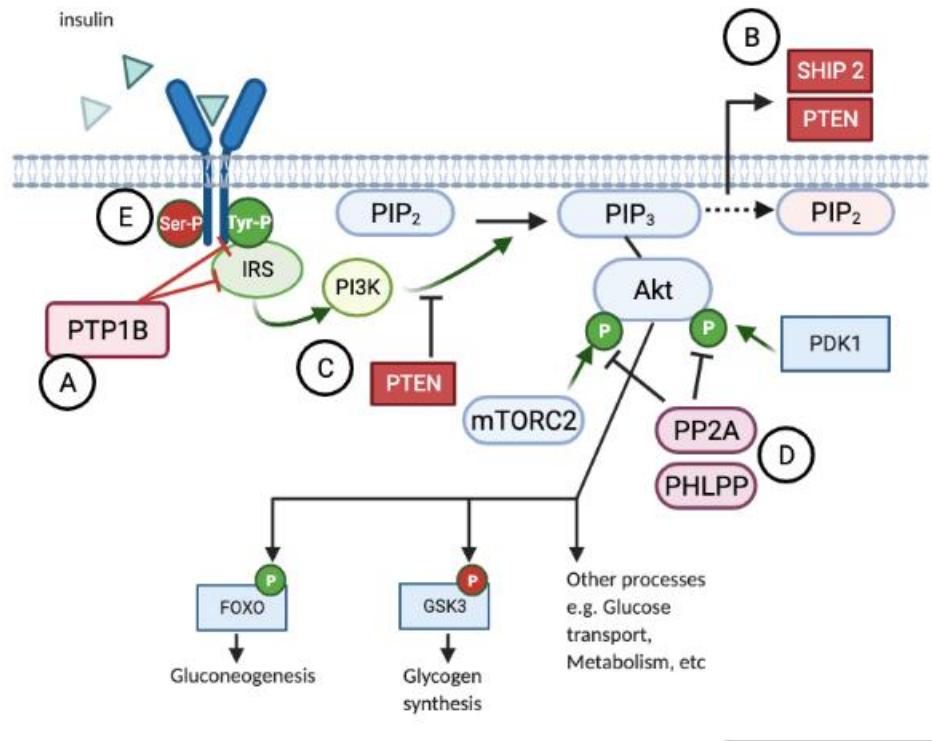


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Figure 1.1 Insulin signalling pathway. Insulin binds the insulin receptor (IR) at the extracellular, alpha subunit. This causes the autophosphorylation of the receptor at key tyrosine residues on the intracellular, beta subunit. Insulin receptor substrate (IRS) proteins are then recruited to the activated IR, acting as a scaffold between the receptor and phosphoinositide 3-kinase (PI3K). Recruitment of PI3K to the IRS proteins leads to the activation of the catalytic subunit of PI3K, causing the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP₂) to generate phosphatidylinositol (3,4,5)-triphosphate (PIP₃). PIP₃ then recruits Akt to the plasma membrane, allowing for phosphorylation at threonine 308 by phosphoinositide-dependent kinase 1 (PDK-1). Furthermore, Akt is phosphorylated at serine 473 by the mammalian target of rapamycin (mTOR) complex 2. When fully activated, Akt can then phosphorylate transcription factors such as forkhead box protein O1 (FOXO1), excluding it from the nucleus and thereby blocking its transcriptional activity and inhibiting the expression of gluconeogenic enzymes, such as PEPCK and G6Pase. Akt can also phosphorylate glycogen synthase kinase 3 (GSK-3) at key serine residues, which in turn allows glycogen synthase (GS) to be active, which means glycogen can be made. Green P indicates activating phosphorylation; Red P indicates inhibitory

The insulin signalling pathway is carefully regulated because it affects a number of critical processes in the body, such as metabolism, cell growth, and differentiation (45). Under normal physiological conditions, insulin signalling is terminated upon dissociation of insulin that results in rapid dephosphorylation of the IR and its substrates (70) as depicted in Figure 1.2A. Several protein tyrosine phosphatases (PTPases) can catalyse these dephosphorylation events (71, 72). However, scientific studies have mainly focused on the cytoplasmic protein tyrosine phosphatase 1B (PTP1B), the negative regulator of the insulin receptor (73–77). Disruption of the gene encoding this enzyme in mice has been shown to improve insulin sensitivity and increases insulin-dependent tyrosine phosphorylation of the IR and IRS proteins in muscle (70). Insulin action can also be temporally controlled by lipid phosphatases that

dephosphorylate PIP₃ (70). An example of such a phosphatase is SHIP2, a phosphoinositide phosphatase, which removes the 5-position phosphate from PIP₃, thereby blocking insulin action through PIP₃ (and not PIP₂) which is responsible for the recruitment of subsequent signalling proteins including AKT (Fig 1.2). Deletion of the SHIP2 protein in mice results in a hypersensitivity to insulin (70). Overproduction of phosphatase and tensin homolog (PTEN) also attenuates insulin signalling by dephosphorylating PIP₃ *in vitro* (78) as well as down-regulating PI3K activity (Fig 1.2) (78–82). Protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPP), are phosphatases responsible for the dephosphorylation of AKT at serine 473 and/or threonine 308 (69, 83) with PHLPP specifically able to dephosphorylate AKT at serine 473 (Fig 1.2) (84, 85). Another mechanism of insulin signalling inhibition is through the serine phosphorylation of the IR as well as IRS proteins (Fig 1.2) (30, 45). An increase in the inhibitory serine phosphorylation of IR and IRS in conjunction with decreased tyrosine kinase activity has been associated with insulin resistance (14, 30, 86). This serine phosphorylation inhibits the interaction of the IR and IRS proteins, thereby inhibiting further downstream action of the insulin signalling pathway (13, 86, 87).



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Figure 1.2 Sites of insulin signalling termination. Multiple sites of regulation exist for insulin signalling. **A)** protein tyrosine phosphatase 1 B (PTP1B) can dephosphorylate tyrosine residues on the insulin receptor (IR) and insulin receptor substrate (IRS) proteins. **B)** The lipid phosphatase SHIP2 and phosphatase and tensin homolog (PTEN) catalyse the dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate (PIP₃) to phosphatidylinositol (4,5)-bisphosphate (PIP₂). **C)** PTEN is also able to downregulate phosphoinositide 3 (PI3)-kinase activity. **D)** Protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPP) can dephosphorylate AKT at its threonine 308 and serine 473 residues. **E)** Inhibitory serine phosphorylation of the IR and IRS proteins.

1.3.1 AKT as the central node of insulin signalling

AKT is a serine/threonine kinase and provides a link between upstream and downstream insulin signalling. As illustrated in Figure 1.1, AKT acts as a central hub for cellular signal transduction induced by insulin, relaying the signal that originated at the cell surface to various intracellular signalling proteins including nuclear proteins. The activation of AKT is initiated when PI3K phosphorylates PIP₂ to generate PIP₃ at the inner plasma membrane. This allows the recruitment of AKT which binds to PIP₃ via its PH domain, anchoring it to the plasma membrane and allowing it to become phosphorylated at its threonine 308 residue found in the activation T-loop, by PDK1, as mentioned earlier (section 1.3) (44, 83, 88–92). In addition, AKT is further phosphorylated at serine 473, which is situated in its hydrophobic domain, by mTOR2 (Figure 1.1) (90, 92–94). As previously mentioned, (section 1.1 and 1.3) phosphorylation at both these sites is integral for the full activation of the kinase activity of AKT (44, 46, 47).

The fully active AKT will translocate from the cell membrane to the cytosol and the nucleus (90), where it is able to phosphorylate numerous downstream substrates responsible for the regulation of a plethora of other cellular processes (44–46, 88, 95).

The cellular processes affected by AKT regarding insulin signalling depends on the cell and tissue type involved. In adipose and skeletal muscle cells, for example, AKT signals for the translocation of the GLUT4 vesicle to the plasma membrane. Once GLUT4 is incorporated in the plasma membrane it allows for the uptake of glucose thereby lowering blood glucose concentrations (47, 63, 96). Meanwhile, in the liver, the main function of insulin mediated by AKT is to decrease blood glucose concentrations by preventing the *de novo* synthesis of glucose. Activated AKT will phosphorylate the transcription factor forkhead box protein O1 (FOXO1), targeting it to be exported from the nucleus (97–100) as well as for proteasomal degradation (101–103). Removal from the nucleus will prevent FOXO1 from regulating the gene expression of key gluconeogenic enzymes, such as G6Pase and PEPCK, thus decreasing hepatic glucose production by gluconeogenesis (101–103). Other targets of AKT in the liver include the phosphorylation of GSK-3 at key serine residues (serine 21 for GSK-3 α and serine 9 for GSK-3 β), thereby inactivating GSK-3 and preventing the deactivation of GS and subsequent inhibition of glycogen synthesis (50). It therefore encourages the storage of glucose in the form of glycogen by increasing glycolysis and glycogenesis (97, 104). AKT is also responsible for the upregulation of sterol regulatory element binding protein 1c (SREBP-1c), the lipogenic transcriptional regulator which promotes *de novo* lipogenesis by augmenting transcription of several lipogenic enzymes (55, 66).

1.3.2 Downstream effects of insulin

Insulin signalling *via* AKT targets multiple cellular processes including gluconeogenesis and glycogen synthesis. These two metabolic processes are especially important in energy metabolism and their dysregulation are commonly associated with insulin resistance (67, 97, 105) and will be discussed next.

1.3.2.1 Downstream effect of insulin: gluconeogenesis

Gluconeogenesis is the process whereby non-carbohydrate substrates (such as glycerol and lactate) are converted to glucose and/or glycogen (23). This metabolic pathway is driven by several enzymes, with the first reaction catalysed by PEPCK and the last reaction in the pathway by G6Pase. Hepatic glucose production takes place in the absence of insulin when the body experiences starvation. This is mainly due to the fact that expression of gluconeogenic

genes is under hormonal control (61, 98, 99, 106–109) and regulated both transcriptionally and translationally (110). Transcription factors essential for gluconeogenesis include cAMP response element binding protein (CREB), and nuclear factors such as the GR and the forkhead box class Os (FoxOs) of which some are inhibited by insulin (CREB and GR being the exceptions) (110). In the absence of insulin and presence of glucagon, binding of glucagon to its cognate receptor activates a signalling cascade that generates cyclic AMP (cAMP) through the activation of adenylyl cyclase. This second messenger in turn stimulates cAMP-dependent protein kinase A (PKA), which subsequently phosphorylates CREB. Phosphorylated CREB then binds to the CREB response element (CRE) in the promoter region of its target genes, including the genes, *PCK1* and *G6PC*, encoding the gluconeogenic enzymes PEPCK and G6Pase, respectively, leading to their increased transcription (111). In the presence of insulin, *de novo* synthesis of glucose is inhibited (66), mainly due to the activation of AKT and subsequent phosphorylation of FOXO1 (101, 102). FOXO1 of course as previously mentioned is another transcription factor required to induce the transcription of *PCK1* and *G6PC* (66, 97). Phosphorylation of FOXO1 by AKT results in its nuclear exclusion, where it can no longer bind to DNA and induce transcription of the gluconeogenic genes. FOXO1 phosphorylation also targets it for proteasomal degradation (101–103). G6Pase is the enzyme responsible for the hydrolysis of glucose 6-phosphate (G-6-P) to glucose, the last step of the metabolic pathways critical to the production of glucose in the liver *via* glycogenolysis and gluconeogenesis (112, 113). Glucose and not G-6-P can enter the bloodstream (114). PEPCK in turn is responsible for the cytoplasmic conversion of oxaloacetate to phosphoenolpyruvate, the rate-limiting step in gluconeogenesis (23). Most metabolic enzymes are regulated either allosterically or *via* posttranslational modification. This is not the case for these two key gluconeogenic enzymes, PEPCK and G6Pase, which are mainly regulated at the transcriptional level (99, 115). The transcriptional regulation of these genes involve complex interactions of a variety of transcription factors such as those mentioned earlier in this section as well as other proteins (99). Besides insulin and glucagon, *G6PC*, and *PCK1* gene expression is also regulated by GCs (23, 98, 111). GCs signals *via* the GR, and like FOXO1 is a transcription factor, which recognises specific DNA sequences known as GREs within the gene promoter of *G6PC* and *PCK1* leading to increased gene expression (98). Ligand-activated GR signalling will be discussed in more detail later in this chapter (section 1.5).

1.3.2.2 Downstream effect of insulin: glycogen synthesis

Excess glucose not needed for energy is stored as glycogen in the muscle and the liver. The breakdown of glycogen increases the amount of glucose available between meals. Glycogen therefore acts as a buffer to help maintain blood-glucose levels since glucose is fundamentally the only source of energy for the brain (except during prolonged starvation). Glucose from glycogen is readily mobilized and is therefore a good source of energy when required immediately (116).

Glycogen levels are controlled by GS and glycogen phosphorylase (117). GS allows for one glucose molecule at a time to combine with glycogen. Two isoforms of this enzyme exist, namely GS-a (dephosphorylated and the active form) and GS-b (phosphorylated and the inactive form). In its active form, the GS enzyme catalyses the rate limiting step of glycogen synthesis (transfer of glucose monomers from UDP-glucose to the terminal branch of the glycogen chain *via* the formation of $\alpha(1 \rightarrow 4)$ glycosidic bonds) (117, 118). As mentioned earlier, GSK-3 is the key regulator in glycogen synthesis and while most kinases are activated by phosphorylation, GSK-3 kinase activity is inhibited *via* AKT-induced serine phosphorylation (serine 21 in GSK-3 α and serine 9 in GSK-3 β) at the N-terminal of the enzyme (50, 119). Therefore, in the presence of insulin an increase in GS activity is observed due to the inability of GSK-3 to phosphorylate GS thereby inhibiting its function (117, 120). In resting cells, GSK-3 in its active state is highly phosphorylated at key tyrosine residues found in the catalytic loop of the enzyme (tyrosine 279 in GSK-3 α and tyrosine 216 in GSK-3 β) and basally active (51). In this way it plays a role as a negative regulator of the insulin signalling pathway (50). Mutation of either of these residues to alanine results in full impairment of GSK-3 kinase activity (48, 121). T2DM is strongly linked to decreased insulin-stimulated GS activity and glycogen synthesis (50, 122–125).

Glycogen phosphorylase, which also regulates glycogen concentration, is the key enzyme in glycogenolysis, responsible for catalysing the rate-limiting step in glycogen breakdown: the hydrolysis of glycogen to generate glucose-1-phosphate and a shortened glycogen molecule. This enzyme, like GS, also exists in two isoforms, namely: phosphorylase a (active and phosphorylated) and phosphorylase b (inactive and dephosphorylated). The important regulators of this enzyme are phosphorylase kinase, which catalyses the phosphorylation reaction (phosphorylase b \rightarrow a) and protein phosphatase, which catalyses the dephosphorylation reaction (phosphorylase a \rightarrow b). Insulin regulates this pathway by activating

protein phosphatase 1, resulting in the inactive glycogen phosphorylase b and therefore decreased glycogen breakdown (117, 120).

1.4 Insulin resistance

1.4.1 Defining insulin resistance

As mentioned earlier, insulin resistance can be briefly described as the inability of insulin target tissues to react to ‘normal’ concentrations of insulin (126). It involves the disruption of specific events in the insulin signalling pathway (127). Consequently, the insulin target tissues require elevated levels of insulin for adequate glucose disposal (55). Development of insulin resistance typically results in a compensatory increase in endogenous insulin production by the pancreas (10, 128).

Risk factors and causes of insulin resistance include obesity (129), an inactive lifestyle, poor diet, chronic inflammation (18, 130–132) as well as chronic stress (10, 127, 133–135). Conditions that present with insulin resistance include central obesity (15, 130), high blood pressure (22, 130), high cholesterol as well as T2DM (the predominant consequence of insulin resistance), all of which fall under the blanket term “metabolic syndrome” (130, 136–139). The ramifications of insulin resistance include, but are not limited to, hyperglycaemia (7, 104, 140, 141), hyperinsulinemia (60, 138, 142), dyslipidaemia (22, 143), visceral adiposity, and elevated inflammatory markers (15, 130–132). Elevated inflammation therefore appears to be both a cause and a result of insulin resistance.

1.4.2 Mechanisms by which insulin resistance can occur

There are a few proposed molecular mechanisms by which insulin resistance occurs. These include either mutations in the IR or other key proteins in the signalling pathway, antagonists of insulin or even reduced expression and/or phosphorylation of the key proteins in the insulin signalling pathway (45, 138, 144–147). Furthermore, termination of insulin signalling also results in insulin resistance. Serine phosphorylation of the IRS molecules are generally involved in negative regulation and interfere with its binding to the IR as these proteins then cannot be phosphorylated at the specific tyrosine residues (13, 14, 45). This then causes reduced docking of PI3K as well as impaired insulin stimulated glucose transport (mainly in muscle and adipose tissue) and decreased glycogen synthesis (primarily in the liver and muscle) as well as increased hepatic glucose production due to decreased insulin-stimulated inhibition of gluconeogenic genes (14, 19, 148–150).

Another mechanism of regulation of the insulin signalling pathway is the phosphorylation and subsequent activation of protein tyrosine phosphatases (e.g. PTP1B), responsible for the dephosphorylation of tyrosine residues of the IR and the IRS or lipid phosphatases (e.g. SHIP2, PTEN, etc) responsible for the dephosphorylation of PIP₃ to PIP₂ leading to the termination of insulin action (70, 76, 78, 82, 148, 151–153). These negative regulators have been shown to be upregulated in an insulin resistant state (154, 155).

In the liver, loss of the IR leads to severe insulin resistance, glucose intolerance, and the inability of insulin to inhibit glucose production (156). Deletion of IRS-1 in mice results in hepatic insulin resistance manifesting as serum hyperinsulinemia (157), while deletion of the IRS-2 protein in mice results in severe hepatic insulin resistance (157) as well as increased gluconeogenesis (158). Furthermore it has been found that inactivating mutations in the SH2 domain of the p85 α regulatory subunit of PI3K (159) or deletion of both the p85 α and p110 α subunits (160) can result in decreased insulin signalling as well as hyperinsulinemia. Although this is not physiologically probable, it highlights the fact that dysregulation of the IR, IRS-1 as well as PI3K contributes significantly to insulin resistance (161).

It is thus clear that disruption of insulin signalling can occur at multiple nodes of the pathway. These disruptions can occur at a singular node or at multiple nodes simultaneously, affecting the amplitude of the inhibitory effect on insulin signalling, leading to an insulin resistant state and subsequent T2DM. Inflammation, which is frequently present in the visceral fat of patients with cardiovascular disease and T2DM, is often associated with the aetiology and disease progression of insulin resistance and T2DM. This is due to inflammatory mediators such as pro-inflammatory cytokines negatively affecting multiple points within the insulin signalling pathway (20, 31, 32, 34, 162–167). In addition, the stress response mediated by various hormones, which include the corticosteroids or GCs is tightly linked to inflammation (168). A hypothesis proposed by P. Black (2002) argues that repeated acute or chronic stress may cause the inflammatory response present in T2DM (168). Thus stress hormones such as cortisol (GCs) together with pro-inflammatory cytokines, could possibly increase the inhibition of insulin signalling leading to T2DM development and progression (168, 169). In the sections to follow, the molecular signalling of GCs and pro-inflammatory cytokines will be discussed in more detail to provide insight into their molecular mechanism of action.

1.5 Glucocorticoids (GCs)

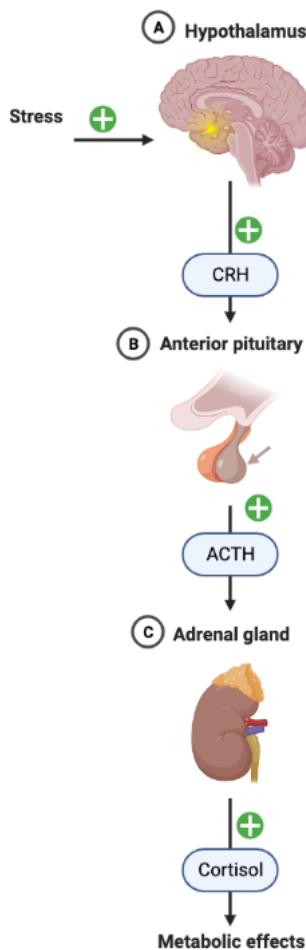
Endogenous GCs (cortisol in humans and corticosterone in rodents) are hormones produced in the adrenal cortex under the control of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.3) (170–172). Under basal or “unstressed” conditions, GCs are released from the adrenal gland (Fig 1.3 –C) in a circadian fashion, with peak concentrations experienced during the active phase (i.e. in the morning for humans). Upon physiological and emotional stress, activity of the HPA axis is further increased (52). Corticotropin-releasing hormone (CRH) is secreted from the hypothalamus (Fig 1.3 –A). CRH then binds its cognate receptor in the anterior pituitary, inducing the release of adrenocorticotrophic hormone (ACTH) as shown in Figure 1.3 -B. ACTH in turn stimulates the adrenal gland to synthesize and secrete cortisol into circulation (Fig 1.3 –C) (173–176). Once in the bloodstream, GCs are bound to and transported by plasma proteins, which keep them biologically inactive (\pm 80-90 % bound to corticosteroid-binding globulin (CBG) and \pm 10 % bound to albumin) (172). Due to their small, lipophilic nature, free or unbound GCs in circulation can freely diffuse across the cell membrane and exert their biological functions. The actual bioavailability of GCs in the cytoplasm is regulated by a careful balance between the active, unbound cortisol and inactive, bound cortisol (172, 177–179). Furthermore, active cortisol can be converted to inactivate cortisone by an enzyme known as 11 β -hydroxysteroid dehydrogenase (11 β -HSD). Two 11 β -HSD isozymes exist, type 1 and 2, with 11 β -HSD1 responsible for catalysis of cortisone to cortisol and 11 β -HSD2 responsible for the reverse reaction) (176, 178).

Synthetic GCs, such as dexamethasone (dex) and prednisolone are produced by the pharmaceutical industry for use as anti-inflammatory agents. They were developed based on the structure of cortisol and mimic the effects thereof (180, 181). Synthetic and endogenous GCs differ in their potency, specificity as well as in the fact that most synthetic GCs do not bind carrier proteins like CBG (172). In terms of potency, synthetic GCs are usually much better activators of the GR than cortisol, mainly due to their higher binding affinity for the GR (172, 182, 183). Where specificity is concerned, endogenous GCs are known to activate both the GR and the mineralocorticoid receptor (MR), whereas synthetic GCs such as dex almost exclusively activate the GR (172, 182, 183). Synthetic GCs like dex are also not subject to metabolic processing by 11 β -HSD1/2 as is the case for endogenous GCs and therefore this has a major impact on the bioavailability of synthetic GCs (172, 182–184). However, their prolonged use is often associated with complications and undesired side effects such as central

obesity, hypertension, and increased hepatic glucose production, which are dependent on the duration and dose of the treatment (185, 186).

GC action is mediated by acting as a ligand for the GR, an intracellular receptor and ligand-dependent transcription factor. The GR belongs to the steroid receptor family, which is part of the nuclear receptor super family (187, 188). Upon activation by ligand, the receptor can act as a transcription factor and alter the expression of its target genes. The GR can bind to specific DNA sequences known as GREs (187). In the absence of ligand, the GR is part of a large heterodimeric complex with several other proteins that dissociates upon ligand binding. Additionally, ligand binding also leads to the formation of GR homodimers and the subsequent translocation into the nucleus where it can regulate the transcription of specific genes (181). Transactivation is mainly believed to occur *via* direct binding of GR homodimers to the GREs within the promoter sequences of GC-responsive genes (172, 188). The ligand-activated GR is also responsible for the repression of multiple genes (188). Ligand-activated GR monomers can also bind to DNA sequences known as negative GREs (nGREs) in the promoter of the target gene (172, 179, 189–193). Furthermore, GR monomers can bind to half-sites on the DNA and if there is another transcription factor binding site nearby, the two elements can act as a composite site, where either positive or negative interaction between the GR and other transcription factor can occur (172, 193–198). Lastly, gene expression can be influenced by direct protein-protein interactions with other DNA-bound transcription factors such as activator protein 1 (AP-1), signal transducer and activator of transcription (STAT), and nuclear factor kappa B (NF κ B), directly altering the ability of these transcription factors to effectively bind DNA, recruit co-factors, and activate or repress gene transcription (188, 193, 199–201). This so-called tethering of the GR to other transcription factors occurs in the absence of direct DNA binding of the GR (202).

The anti-inflammatory activity of the GR is generally attributed to the repression of pro-inflammatory genes by tethering to transcription factors such as AP-1 and NF κ B thereby blocking their transcriptional activation of pro-inflammatory genes (described further in section 1.6) (193, 199, 203–206). The anti-inflammatory nature of GCs are also coupled to augmented anti-inflammatory cytokine expression by direct binding of the GC-bound GR to GREs in the promoter region of several anti-inflammatory genes (43).



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Figure 1.3 The stress response/ HPA axis. **A)** Stress stimulates the hypothalamus to release corticotropin-releasing hormone (CRH). **B)** CRH then acts on the anterior pituitary gland to stimulate the synthesis of adrenocorticotrophic hormone (ACTH). **C)** ACTH in turn, acts on the adrenal cortex to induce the secretion of glucocorticoids (such as cortisol in humans) from the adrenal cortex. Once secreted, they are able to exert a variety of tissue-specific metabolic effects. Green plus indicates increase.

1.5.1 Glucocorticoids and insulin resistance

GCs have longed been associated with T2DM and insulin resistance (24, 186, 188). In fact the effect this adrenal steroid has on glucose metabolism and blood glucose levels influenced its fitting and explanatory name (207). For example, a study done by Yan, *et al.*, concluded that chronic stress was associated with insulin resistance and may further contribute to the development thereof (208).

A well-known metabolic effect of GCs is the upregulation of gluconeogenesis consequently leading to increased hepatic glucose production (23, 186, 209–212). The ligand-activated GR is the transcription factor responsible for the gene regulation of *G6PC* and *PCK1* (52, 98, 109, 209, 213–215). The *G6PC* and *PCK1* promoter activation induced by GCs leads to an increase in the mRNA and protein levels of these enzymes (186, 216, 217).

However, GCs are also capable of influencing glucose metabolism *via* interfering with insulin signalling more directly (218, 219). GCs have been proven to decrease protein expression of IRS-1 in rat muscle (220, 221) and adipocyte cell lines (222) as well as decrease tyrosine phosphorylation of the IR and IRS-1 in muscle cells (220). Similarly, a study by Giorgino, *et al.*, proved that GC-treated rat muscles showed a decrease in tyrosine phosphorylation of IR due to a decrease in the total IR protein (223). A study by Miao, Zhang, Lu, *et al.*, observed an increase in serine phosphorylation of IRS-1 following GC treatment in liver cells (224). The increase in transcription of PTP1B in response to GC treatment has also been demonstrated in rat muscle (221).

Furthermore, treatment with GCs have been shown to decrease AKT protein expression (17) as well as phosphorylation at both the threonine 308 and serine 473 sites in adipocytes and muscle of rats (17) as well as in their mast cells (218).

Downstream of AKT, GCs have been proven to affect insulin signalling by reducing the insulin-induced GSK-3 β inhibitory phosphorylation at serine 9 (17). The authors of this study, however, did not investigate the phosphorylation status of the tyrosine residues responsible for maximal GSK-3 activity. Nonetheless, the presence of increased concentrations of GCs for extended periods of time could lead to disrupted insulin signalling, decreased glycogen synthesis as well as increased gluconeogenesis.

As mentioned in section 1.4.1, obesity is both a cause of, as well as a condition that presents with, insulin resistance. It is also a potential link between GC- and inflammation-mediated insulin resistance (132) as obesity (characterised by chronic low-grade inflammation) is a known side effect of chronic overexposure to GCs (28, 133, 225, 226).

1.6 Inflammation and pro-inflammatory cytokines

The inflammatory response can be described as the concerted activation of signalling pathways that control inflammatory mediator levels in the resident tissue cells and inflammatory cells recruited from the blood. Uncontrolled and sustained acute inflammation is a common pathogenesis of many chronic diseases including cardiovascular disease and diabetes, among others (227). The inflammatory response is an important part of immunity (228). Obesity, a key risk factor in the development of T2DM as well as a side effect of long-term GC use, is characterised by chronic low-grade inflammation – increased levels of pro-inflammatory cytokines such as TNF- α and IL-6 in circulation (229–231). These cytokines are able to interfere with normal insulin function and subsequently assist in the development of insulin

resistance (21, 232). The term ‘cytokine’ is the general name for polypeptides secreted by cells in an effort to upregulate the inflammatory response. They promote systemic inflammation (233–235). TNF- α and IL-6 are early release cytokines that are mainly associated with acute inflammation (233). However, prolonged exposure to these pro-inflammatory cytokines due to a persistent acute inflammatory response, observed with obesity, often results in disrupted insulin signalling leading to insulin resistance (225, 227, 233). For this reason, the signalling pathways of these two pro-inflammatory pathways will be briefly described in the following sections.

1.6.1 Tumour necrosis factor alpha (TNF- α)

TNF- α is a potent pro-inflammatory cytokine involved in the stimulation of inflammation, particularly an acute phase immune response (228, 236). It is also the first cytokine to have been reported to represent a link between obesity, inflammation, and diabetes (19, 21). TNF- α exerts its biological effects by binding to two plasma membrane receptors, TNF-receptor 1 (TNF-R1) and TNF-receptor 2 (TNF-R2) with the majority of the biological effects mediated *via* TNF-R1 (237). TNF-R1-mediated signalling leads to the activation of the transcription factor NF κ B (237). NF κ B is normally sequestered in the cytoplasm by members of the inhibitor of κ B (I κ B) protein family. TNF- α -induced signalling to NF κ B involves the activation of I κ B kinase (IKK). Once activated, the IKK β subunit of IKK phosphorylates the IKK α subunit, which leads to its ubiquitination and subsequent proteasomal degradation. This allows NF κ B to translocate into the nucleus where it can activate transcription of NF κ B responsive genes by binding to κ B sites situated within their promoter regions (237). NF κ B responsive genes that are upregulated by binding of NF κ B to the κ B site include IL-6 (238, 239), numerous acute phase proteins involved in the acute phase response (240–245), as well as PTP1B (76). Genes repressed or downregulated by NF κ B signalling include *G6PC*, encoding the gluconeogenic enzyme G6Pase (112, 246), and *HSD11B2*, which encodes for 11 β -HSD2 that is responsible for the conversion cortisone to cortisol (247).

1.6.2 Interleukin-6 (IL-6)

Like TNF- α , IL-6 has also been identified as an important mediator contributing to insulin resistance (167). Additionally, and similarly to TNF- α , IL-6 is also an important inducer of the acute phase response (163, 168, 248). IL-6 is synthesised and secreted during inflammatory

conditions upon stimulation by TNF- α (163) and its serum levels positively correlate with the degree of obesity and the development of T2DM (163, 249). Two mechanisms of IL-6 signalling exists: classic signalling and trans-signalling. IL-6 binds the IL-6 receptor (IL-6R), which on its own is not signalling competent. The classical signalling pathway is initiated upon the association of the IL-6/IL-6R complex with a second receptor, named glycoprotein 130 (gp130). Gp130 is expressed on all cells of the body whereas IL-6R is only expressed on certain cell types including hepatocytes. Therefore, only IL-6R expressing cells can directly respond to IL-6, leading to classic signalling. Trans-signalling involves the use of a soluble IL-6 receptor (sIL-6R), which is then able to bind gp130 and initiate signalling in cells that do not express the membrane bound IL-6R. Gp130 dimerization, upon binding of the IL-6/IL-6R complex, leads to the activation of the tyrosine kinase, JAK1, which is constitutively bound to the cytoplasmic portion of gp130. After autophosphorylation, JAK1 phosphorylates tyrosine residues within the cytoplasmic portion of gp130, leading to the activation of several intracellular signalling pathways including the MAPK, PI3K, and the STAT 1 and 3 pathways (250–252). STAT3, a transcription factor for example, in the liver suppresses the *PCK1* and *G6PC* genes (58, 253).

1.6.3 Inflammatory cytokines and insulin resistance

Pro-inflammatory cytokines acting *via* their respective receptors initiate a cascade of molecular events as described above, which could influence insulin signalling. STAT3 for example, which is activated by IL-6, is also able to upregulate suppressor of cytokine signalling (SOCS) 3, a negative regulator of cytokine signalling (163), which has also been shown to inhibit insulin signalling by directly binding to the phosphotyrosine 960 of the IR *via* its SH2 domain. It therefore prevents IRS1 and IRS2 interaction with the IR (31). Furthermore, the expression of SOCS-3 also inhibits autophosphorylation of the IR (34, 163–166). Inhibition of tyrosine phosphorylation, by pro-inflammatory cytokines such as TNF- α (164) as well as IL-1 β (32), of IR and IRS proteins in the murine adipocyte 3T3-L1 cells have also been reported. In addition, pro-inflammatory cytokines such as IL-1 β (32) and IL-6 (167) have been shown to decrease phosphorylation of AKT at threonine 308 (32) and serine 473 (167). Meanwhile it has also been reported that TNF- α induces serine phosphorylation of the IR and IRS-1 through activation of p38 MAPK and IKK, which in turn impairs its tyrosine phosphorylation by insulin and the subsequent activation of PI3K and AKT in rat myotubes (162). The authors reported that the increase in IR serine phosphorylation led to a decrease in glucose uptake due to decreased GLUT4 translocation, a hallmark of an insulin resistant state (162).

It is not only the phosphorylation status of insulin signalling mediators that are negatively influenced by pro-inflammatory cytokines. The protein levels of key nodes within the insulin signalling pathway are also affected. For example, the pro-inflammatory cytokine IL-1 β was shown to decrease IRS-1 protein expression in 3T3-L1 cells (32). Both IL-6 and TNF- α decreased the mRNA expression and subsequently the protein expression of IRS-1 in 3T3-L1 cells (254). Downstream, Medina, *et al.*, demonstrated that treatment of 3T3-L1 cells with TNF- α resulted in decrease AKT levels (255).

Further downstream effects of insulin are also negatively regulated by pro-inflammatory cytokines such as TNF- α and IL-6. Senn, *et al.*, reported that primary hepatocytes treated with IL-6 led to a 75% reduction in insulin-induced glycogen synthesis (34). The inflammatory cytokine, TNF- α has also been shown to induce PTP1B protein expression, which negatively affects insulin signalling. Furthermore, treatment with TNF- α resulted in the increased expression of PTP1B in 3T3-L1 adipocytes as well as the H4IIE a hepatoma cell line (76). In contrast, the pro-inflammatory cytokine IL-6, has been implicated in the negative regulation of G6Pase in a STAT-dependent manner *in vitro* and *in vivo* (256–258) thereby inhibiting the *de novo* production of glucose similar to the function of insulin.

To summarise, pro-inflammatory mediators such as TNF- α and IL-6, like GCs, can influence insulin signalling at various nodes within the pathway, which could contribute to a reduction in the action of insulin potentially increasing the severity of an insulin resistance pathology as well as the rate at which it manifested.

1.7 Evidence of co-regulation

As previously mentioned, GCs and pro-inflammatory cytokines are traditionally known for their opposing molecular actions. GCs are often prescribed to patients combating inflammation as it is well-known for its anti-inflammatory action. However, as detailed in the previous sections, prolonged exposure to GCs or pro-inflammatory cytokines results in the pathogenesis of insulin resistance and subsequent T2DM.

To the best of our knowledge, whether concomitant exposure to both these insulin resistant causative agents would influence insulin signalling, is unknown. GCs and pro-inflammatory cytokines acting in a co-operative manner, however, is not a novel concept. Acute phase protein expression appears to be especially favoured to be co-regulated by these signalling molecules.

A number of studies have demonstrated that GCs in the presence of pro-inflammatory cytokines such as IL-6 or TNF- α upregulate the expression of positive acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) (244, 259, 260). Liu, *et al.*, demonstrated that dex in the presence of either TNF- α or IL-6 works co-operatively to increase the expression of the murine acute phase protein SIP24/24p3 above levels induced by either of the three compounds on their own in 3T3-L1 murine adipocytes (261). Furthermore, another study showed that treatment of HepG2 cells with dex and IL-6 simultaneously increased CRP accumulation beyond that of IL-6 or dex only treatments (262). There also exists evidence for the enhancement of SAA transcription when co-treated with dex and either IL-1 β or IL-6 compared to either of the compounds alone (259). These results remain important as SIP24/24p3, CRP, and SAA are all acute phase proteins, which have been shown to be elevated in and even contribute to an insulin resistant state (263, 264).

Thus, evidence does exist whereby co-regulation by GCs and pro-inflammatory cytokines have been reported, although primarily for acute phase proteins, indicative of GCs assisting with the initial stages of the inflammatory response. It would be interesting to examine whether co-regulation of insulin signalling also occurs as both GCs and cytokines have been reported to individually negatively influence insulin signal transduction.

1.8 Aims of this study

From the literature described above it is clear that GCs and inflammatory mediators such as TNF- α and IL-6 negatively affect insulin signalling. However, what is not clear is whether these signalling mediators concomitantly disrupt insulin signalling, which could explain the insulin resistance pathology observed in chronic stress and inflammatory conditions. For this reason, the current study ventured to determine whether the GCs, dex, corticosterone and cortisol and the pro-inflammatory cytokines, TNF- α and IL-6, affect various nodes within insulin signal transduction. More specifically, the effect of these cell mediators on insulin signalling in the liver was investigated using murine- and human hepatoma cell lines, BWTG3 and HepG2, respectively. These cell lines served as an *in vitro* cell model system to study hepatic insulin signal transduction.

Thus, the aims of the current study were as follows:

1. To determine whether GCs and the pro-inflammatory cytokines, co-regulate the protein expression of AKT, the central molecule involved in insulin signalling.
2. Whether co-treatment of GCs and cytokines affects AKT phosphorylation at two key regulatory residues, threonine 308 and serine 473.
3. To determine if downstream of the insulin signalling pathway, co-treatment with GCs and the pro-inflammatory cytokines affect the protein expression as well as tyrosine phosphorylation of GSK-3
4. To determine whether GCs and pro-inflammatory cytokines co-regulate the expression of G6Pase, a key gluconeogenic enzyme.

In Chapter 2 of this thesis the material and methods used will be described, whilst the results obtained in the study will be presented in Chapter 3. The discussion and conclusion of the current study will be reported in Chapter 4, placing the findings of the current study within the context of the literature. In addition, Chapter 4 will also include any future studies which could possibly follow from the current study.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Test compounds

The GCs dex, corticosterone (Cort) and hydrocortisone (F) were obtained from Sigma-Aldrich, dissolved in absolute ethanol and stored in glass vials at -20°C. TNF- α and IL-6 were purchased from Thermo Fisher Scientific and reconstituted in 100 nM acetic acid. Cytokines were subsequently stored at -20°C in supplemented Dulbecco's Modified Eagle's Medium (DMEM) as described below.

2.2 Mammalian cell culture

2.2.1 Cell growth and maintenance

The mouse hepatoma cell line, BWTG3, was a generous gift from Guy Haegeman at the University of Gent, Belgium (265, 266). The human hepatoma cell line, HepG2, was purchased from Cellonex. Both cell lines were cultured in DMEM containing 1000 mg/l glucose and supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich) and 1% Penicillin/Streptomycin (Sigma-Aldrich). All cells were maintained at 37°C in 75 cm² tissue culture flasks at a relative humidity of 90% and 5% CO₂. Cells were tested for mycoplasma infection quarterly to ensure that only mycoplasma negative cells are used in all experiments. No mycoplasma infection was detected during the course of this study.

2.2.2 Treatment conditions

Cells were seeded using the culture medium as described above, into the appropriate vessels depending on the type of experiment. For western blot analysis, cells were seeded at a density of 2x10⁵ cells per well in 12-well plates (BioSmart Scientific). For total RNA isolation, cells were seeded at a density of 5x10⁵ cells per well in 6-well plates (BioSmart Scientific). Seeding in 6-well plates yielded a better total RNA yield than what was observed when using 12-well plates. After 24 hours, supplemented DMEM was removed and replaced with serum- and antibiotic-free DMEM for 24 hours to ensure any activation of the insulin signalling pathway was attributed to that of insulin treatment only and not due to that of the traces of insulin and growth hormones present within the FCS. Following this, cells were treated with either a GC, a cytokine alone or a combination of a GC and a cytokine for 24 hours (Table 2.1). The vehicle control was a combination of 0.1% ethanol and 0.1% supplemented DMEM as these were what the GCs and cytokines were prepared in, respectively. Insulin stimulation was done 30 minutes before the cells were lysed.

Table 2.1. Table indicating the treatment conditions for experiments done in this study

	20 ng/ml TNF-α	20 ng/ml IL-6
10 nM GC	10 nM GC + TNF- α	10 nM GC + IL-6
100 nM GC	100 nM GC + TNF- α	100 nM GC + IL-6
1 μ M GC	1 μ M GC + TNF- α	1 μ M GC + IL-6

Key:

	Glucocorticoid
	Cytokine
	Co-treatment

2.3 Total RNA extraction

Cells were maintained and treated as described in sections 2.2.1 and 2.2.2 respectively. Total RNA was extracted using TRIzol Reagent (Invitrogen), following the manufacturer's instructions. Briefly, 400 μ l of TRIzol was added to each well, making sure the TRIzol covers the surface of the entire well. To ensure adequate lysis of the cells, the plates were stored at -20°C overnight. After thawing on ice, the contents of each well were transferred to a clean, RNase-free 1.5ml microcentrifuge tube and 80 μ l of chloroform was added to each of the samples. The samples were then vortexed for 1 minute and then centrifuged at 14 000 x g for 15 minutes at 4°C. After centrifugation, 140 μ l of the clear aqueous phase (containing the RNA) was transferred to a clean, 1.5 ml RNase-free microcentrifuge tube and 200 μ l of ice-cold isopropanol was added to each microcentrifuge sample. The samples were again vortexed for 1 minute and then stored at -20°C for a minimum of 3 days in order to maximise RNA precipitation. The samples were then centrifuged at 14 000 x g for 30 minutes at 4°C to allow the precipitated RNA to form a pellet. The supernatant was then discarded, and the pellets

washed by adding 500 µl of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated RNA-grade water to the microcentrifuge tube containing the pellet. The samples were then vortexed for 1 minute. This was followed by centrifugation at 14 000 x g for 5 minutes at 4°C. This wash step was repeated at least twice to ensure adequate removal of contaminants. After washing the pellet, the supernatant was discarded, and the pellet centrifuged to reach maximum speed, mimicking the action of a picofuge. Any remaining liquid was removed by pipetting and the pellet left to air dry for 10 minutes. The pellet was then dissolved in 20 µl DEPC-treated RNA-grade water and placed on a heating block at 55°C for 5 minutes. The RNA concentration was then measured using a Nanodrop ND-1000 spectrophotometer. The integrity of the RNA was assessed on a 1% (w/v) agarose gel stained with Nancy-520 (267) (Sigma-Aldrich) to confirm the presence of undamaged 28S and 18S ribosomal bands. RNA samples were then stored at -20°C for later use.

2.4 Complementary DNA (cDNA) synthesis from mRNA

Complementary DNA was synthesized using the ImProm-II reverse transcription kit (Promega) as per the manufacturer's instructions. Briefly, 2 µg of total RNA was added to 50 µg/ml oligo(dT) primers. Nuclease-free water was added to make up the reaction mixture to 5 µl in a thin walled, RNase/DNase-free microcentrifuge tubes for cDNA synthesis (Quality Scientific Plastics). This mixture was then heated at 70°C for 5 minutes. To this mixture was then added a master mix containing 7.6 µl of nuclease-free water, 4 µl of 5x reaction buffer, 1.5 mM MgCl₂, 0.67 mM dNTPs, 20 U of recombinant RNasin® ribonuclease inhibitor, and 160 U ImProm-II reverse transcriptase. The reaction mixture was then placed at 25°C for 5 minutes to allow for primer annealing, then at 45°C for 1 hour for reverse transcription to take place and finally at 70°C for 15 minutes to terminate the reaction by inactivating the reverse transcriptase enzyme. The cDNA samples were then cooled on ice and stored at -20°C for future analysis.

2.5 Quantitative real-time polymerase chain reaction (qPCR)

All qPCR experiments were performed using the LightCycler® 96 (Roche Applied Science) according to the manufacturer's instructions. The qPCR reaction mixture contained 6.25 µl of 1x KAPA SYBR FAST qPCR Master Mix, containing all PCR reagents needed for the PCR including the DNA polymerase enzyme (Kapa Biosystems), 0.5 µM of the respective forward

and reverse primers and nuclease-free water to complete the reaction mixture to 9 µl per sample. This was then added to 1 µl of cDNA or nuclease-free water (non-template control). The PCR conditions used were set using the LightCycler® 96 software. The conditions were as follows: cDNA was pre-incubated at 95 °C for 3 seconds. This was followed by amplification which consisted of denaturation at 95 °C for 3 seconds, primer specific annealing temperature for 20 seconds and extension of DNA at 72 °C for 3 seconds, all repeated 45 times. A melting step at 95 °C for 10 seconds then followed. A 3% agarose gel was then used to verify the size of the product compared to a 100bp DNA ladder as the expected product size was 141 bp (Table 2.2). This was only done for the human G6Pase gene as the rest have been verified previously (264). For BWTG3 samples, GAPDH was used as a reference gene and for HepG2 samples, 18S was used as a reference gene. The mRNA levels of the gene of interest were normalized relative to the respective reference gene. Relative quantification of the target genes was performed using the comparative CT method (delta-delta CT method) with the respective vehicles arbitrarily set to 1. Table 2.2 shows further details specific to each primer pair.

Table 2.2 Human (*Homo sapien*) and mouse (*Mus musculus*) primer sequences as determined using the NCBI website.

<u>Species</u>	<u>Primer name</u>	<u>Sequence (5'→3')</u>	<u>Strand</u>	<u>Amplicon length</u>	<u>Annealing temperature</u>	<u>Primer efficiency</u>
Human	G6Pase	TGCAAGGGAGAACTCAGCAA	Forward	141 bp	54° C	1.92
		GGACCAAGGAAGCCACAATG	Reverse			
	18S	ACATGCCGCCTGGAGAACCT	Forward	181 bp	60° C	2.05
		GCCCAGGATGCCCTTAGTGG	Reverse			
Mouse	G6Pase	TGCAAGGGAGAACTCAGCAA	Forward	64 bp	60° C	1.69
		GGACCAAGGAAGCCACAATG	Reverse			
	GAPDH	GAGAACGGCTACCACATCCAAG	Forward	90 bp	60° C	2.08
		CCTCCAATGGATCCTCGTTA	Reverse			

2.6 Western blot analysis

2.6.1 Preparation of protein lysates

After maintaining and treating cells as described in sections 2.2.1 and 2.2.2 above, the cells were washed with ice cold PBS. The cells were then lysed in 100 µl of passive lysis buffer (0.09 M Tris pH 8.0, 0.5 M EDTA, 10% glycerol, 0.2% (v/v) TritonX-100) containing the following cocktail of protease and phosphatase inhibitors: phenylmethylsulfonyl fluoride (PMSF) – a serine protease inhibitor; sodium fluoride (NaF) – a serine/threonine protein phosphatase inhibitor and sodium orthovanadate (Na₃VO₄) – a protein tyrosine phosphatase inhibitor. Lysates were then transferred to clean 1.5 ml microcentrifuge tubes and 100 µl of 1 x sodium dodecyl sulphate (SDS) sample buffer (1 M Tris-HCl, pH 8.6; 10% (v/v) SDS; 0.1% (w/v) bromophenol blue; 2% (v/v) β-mercaptoethanol and 20% (v/v) glycerol) was added to the lysates. Samples were then boiled for 10 minutes at 95 °C to facilitate denaturation of the proteins.

2.6.2 SDS- polyacrylamide gel electrophoresis and western blot

Protein lysates were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% SDS polyacrylamide gel. Proteins were electrophoresed at 100V for 15min and then at 200V for 90 minutes. After electrophoresis, the proteins were transferred to 0.45 μ M nitrocellulose membrane (Amersham) at 0.18 A for 2 hours. The membranes were then blocked for 90 minutes at room in either 5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) in tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl) containing 0.1% Tween20 (TBST) or 5% (w/v) instant skimmed milk powder in TBST. After blocking, the membranes were then washed with TBST, twice for 5 minutes each, and then with TBS for 5 minutes. This was followed by incubation of the membrane in the appropriate primary antibodies (Cell Signalling Technology or Santa Cruz Biotechnology) described in Table 2.3 overnight at 4°C. The membranes were then washed again as described above before being incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology or Sigma-Aldrich) diluted in 1% (w/v) BSA in TBST or 5% (w/v) instant skimmed milk powder in TBST, described in Table 2.3, at room temperature for 90 minutes. The membranes were once again washed as described above and then visualised using Clarity™ ECL western blotting substrate (Biorad) and the Thermo Scientific MyECL imager (Thermo Fisher Scientific). Quantification of the bands was performed using the ImageJ programme (NIH) and overlaying of the blots to verify protein size was performed using MyImageAnalysis software (Thermo Fisher Scientific).

Table 2.3 Dilutions and information of primary and secondary antibodies used for western blot analysis

	Primary antibody + catalog no.	Dilution	Secondary antibody	Dilution
Phosphorylated proteins	Phospho – Akt (Ser 473) #4060 (blocked in 5% BSA in TBST)	1:700	Anti-rabbit IgG A9169	1:1000 (diluted in 1% BSA in TBST)
	Phospho – Akt (Thr 308) #13038 (blocked in 5% instant skimmed milk powder in TBST)	1:700	Anti-rabbit IgG A9169	1:1000 (diluted in 5% instant skimmed milk powder in TBST)
	Phospho-GSK-3α/β sc-81496	1:500	Goat anti-mouse IgG-HRP sc-2005	1:10 000 (diluted in 5% instant skimmed milk powder in TBST)
Non-phosphorylated proteins	Akt #9272 (blocked in 5% instant skimmed milk powder in TBST)	1:1000	Anti-rabbit IgG A9169	1:1000 (diluted in 5% instant skimmed milk powder in TBST)
	GSK-3α/β sc-7291	1:500	Goat anti-mouse IgG-HRP sc-2005	1:10 000 (diluted in 5% instant skimmed milk powder in TBST)
Loading controls	EF-1 α1 sc-21758 (blocked in 5% instant skimmed milk powder in TBST)	1:1000	Goat anti-mouse IgG-HRP sc-2005	1:10 000 (diluted in 5% instant skimmed milk powder in TBST)
	HSP90α/β sc-13119 (blocked in 5% instant skimmed milk powder in TBST)	1:5000	Goat anti-mouse IgG-HRP sc-2005	1:10 000 (diluted in 5% instant skimmed milk powder in TBST)

2.7 Statistical analysis

GraphPad Prism® version 8 was used for statistical analysis of data. Student's t-test with Tukey's Multiple Comparison's post-test was used to compare the treatments to one another. Statistical significance was indicated by * where $p<0.05$. No statistical significance was indicated by (ns), where $p>0.05$. All data shown represents at least three independent experiments with error bars indicating standard error of the mean.

CHAPTER THREE: RESULTS

Although glucocorticoids (GCs) and pro-inflammatory cytokines are known to antagonise each other's biological function especially in regard to inflammation, both types of cell signalling mediators have been implicated in the development of insulin resistance and subsequent Type 2 diabetes (T2DM) (136, 179, 268–270). Patients suffering from rheumatoid arthritis for example are more likely to develop insulin resistance (271). Rheumatoid arthritis is associated with chronic systemic inflammation characterised by high tumour necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) levels while in addition exogenous GC treatment is prescribed to combat inflammation (272–275). Despite the association between the development of insulin resistance and chronic systemic inflammation or chronic exposure to GCs, either endogenous or exogenous, very little is known about whether pro-inflammatory mediators such as TNF- α and IL-6 together with GCs affect the development of insulin resistance. It is for this reason that the primary aim of this study was to investigate whether GCs, dex (representing synthetic GCs), corticosterone (mouse endogenous GC) and cortisol (human endogenous GC), and two well-studied pro-inflammatory cytokines, TNF- α and IL-6, co-regulate insulin signalling. This was achieved by investigating the effects of the GCs at three different concentrations in the absence and presence of either of the two pro-inflammatory cytokines on a key node of the insulin signalling pathway, namely AKT.

Total protein expression as well as activation by means of phosphorylation at the two key residues (threonine 308 and serine 473) of AKT were examined using western blot analysis. The inability of AKT to become phosphorylated in response to insulin treatment is commonly associated with an insulin resistant state (44, 141, 276). In addition, the effects of the selected GCs and pro-inflammatory cytokines on the glycogen synthesis node of the insulin signalling pathway was also examined. This was achieved by investigating the protein expression of GSK-3 α and GSK-3 β in response to treatment with the selected GCs, at 1 μ M only, in the absence and presence of either of the two pro-inflammatory cytokines. Additionally, the phosphorylation of GSK-3 α and GSK-3 β (tyrosine 279 for GSK-3 α and tyrosine 216 for GSK-3 β) was also studied in response to the test compounds. Phosphorylation of GSK-3 α at tyrosine 279 and GSK-3 β at tyrosine 216 is indicative of protein stability as well as biologically active GSK-3, unlike phosphorylation at specific serine residues, which inhibits GSK-3 activity (49–51, 119).

Finally, whether GCs and the pro-inflammatory cytokines could co-regulate the inhibitory gluconeogenic node of the insulin signalling pathway was examined. The mRNA expression of glucose-6-phosphatase (G6Pase), an important gluconeogenic enzyme, was investigated in

response to dex, chosen as a representative GC, at three different concentrations in the absence and presence of either of the two pro-inflammatory cytokines.

3.1 Effects of the test compounds on the protein expression of AKT

AKT plays a critical role in the insulin signalling pathway. It is often thought of as the central node of insulin signalling from which numerous other signalling responses branch from (92) as indicated in Figure 1.1 (Chapter 1, section 1.3). Inhibition of insulin-induced AKT signalling is often associated with an insulin resistant state (44, 94, 141, 277) highlighting its importance when studying insulin signalling and insulin resistance. In this study 100 ng/ml insulin had no effect on the protein expression of total AKT in both the BWTG3 and HepG2 cell lines (Figs 3.1A & B, 3.2A & B, 3.3A & B, 3.4A & B). Furthermore, the addition of 20 ng/ml of either TNF- α (Figs 3.1A, 3.2A, 3.3A, 3.4A) or IL-6 (Figs 3.1B, 3.2B, 3.3B, 3.4B) to the insulin treatment did not affect the protein expression of total AKT in both cell lines used. All subsequent treatments with the test compounds were done in the presence of 100 ng/ml insulin.

In the BWTG3 cell line, the synthetic GC, dex at all the concentrations tested had no effect on total AKT protein expression either in the absence (Fig 3.1.C & D) or presence of TNF- α (Fig. 3.1.C). Similarly, lower dex concentrations in the presence of IL-6 did not affect total AKT protein levels, however a slight but significant ($p<0.05$) increase was observed with 1 μ M dex in the presence of IL-6 (Fig. 3.1.D). Unlike with the synthetic GC, total AKT protein levels significantly ($p<0.05$) decreased when BWTG3 cells were treated with corticosterone in the absence of a pro-inflammatory cytokine (Fig 3.2.C & D). Treatment with 100 nM and 1 μ M corticosterone in the presence of TNF- α however, caused a slight but significant ($p<0.05$) increase in total AKT protein expression, which was significantly ($p<0.05$) higher than the insulin only treatment (Fig 3.2.C). Unlike, TNF- α , IL-6 had no significant ($p>0.05$) effect on the ability of corticosterone to decrease total AKT protein expression (Fig. 3.2.D). Total AKT protein levels were restored to basal levels when BWTG3 cells were co-treated with 1 μ M corticosterone and IL-6 in the presence of insulin, although the response was not significantly ($p>0.05$) different to that of 1 μ M corticosterone alone.

In the human hepatocyte cell line (HepG2 cells), dex significantly ($p<0.05$) increased total AKT protein expression when compared to the insulin only treatment. (Fig. 3.3C & D). In the presence of TNF- α , there was no significant ($p>0.05$) difference to dex-only treatments (Fig. 3.3.C. Dex lost its ability to increase total AKT protein levels when co-treated with IL-6 (Fig.

3.3D)), with a significant ($p<0.05$) decrease in AKT protein levels at all concentrations of dex in the presence of IL-6. In contrast to dex treatment, the endogenous human GC, cortisol had no significant ($p>0.05$) effect on total AKT protein levels in the absence or presence of the pro-inflammatory cytokines (Fig. 3.4.C & D).

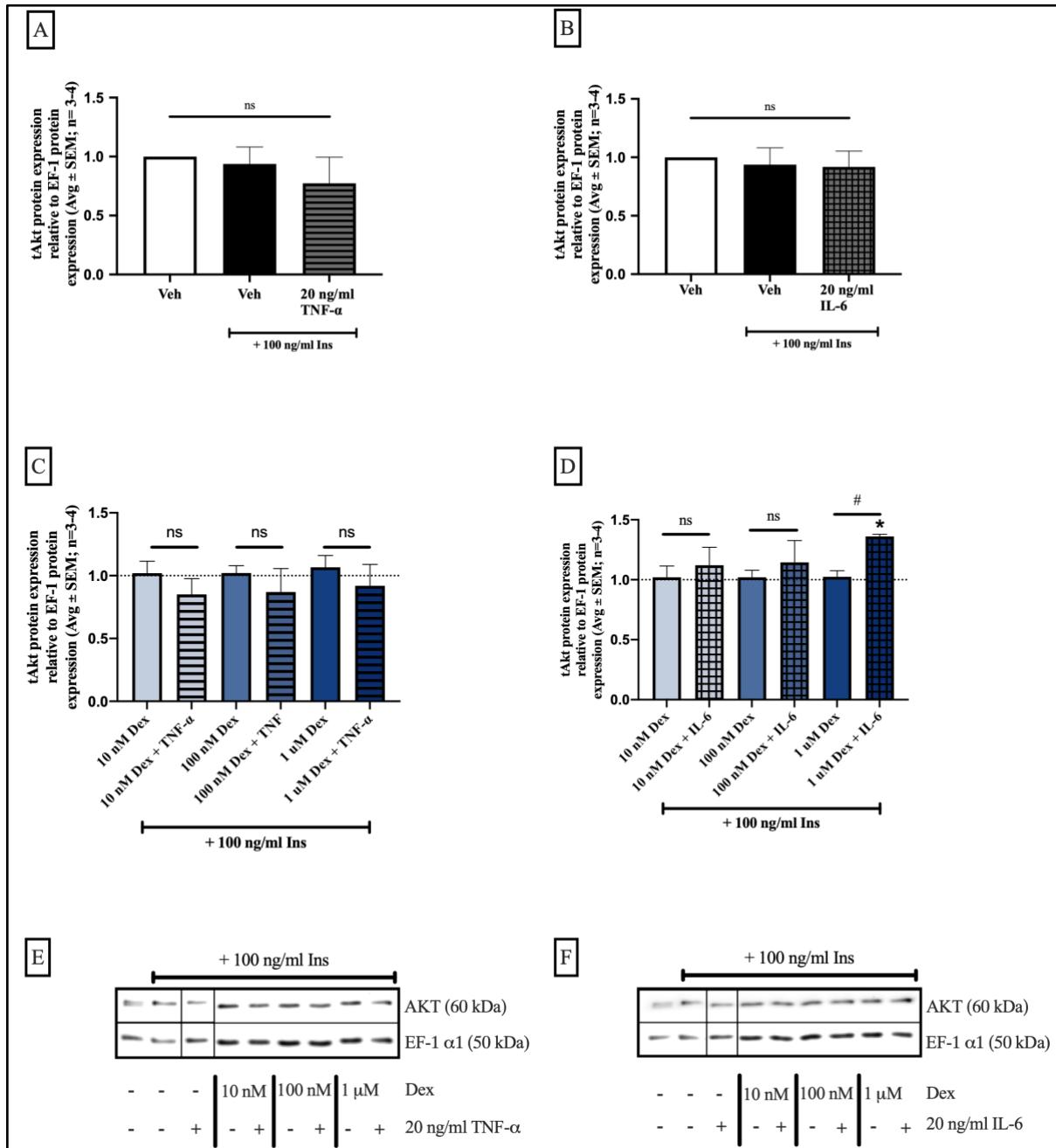


Figure 3.1. Dex only at the highest concentration tested in the presence of IL-6 increases total AKT protein expression in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dex, or 20 ng/ml of either TNF- α or IL-6. For co-treatments, 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Total AKT expression was measured and quantified using EF-1 α as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

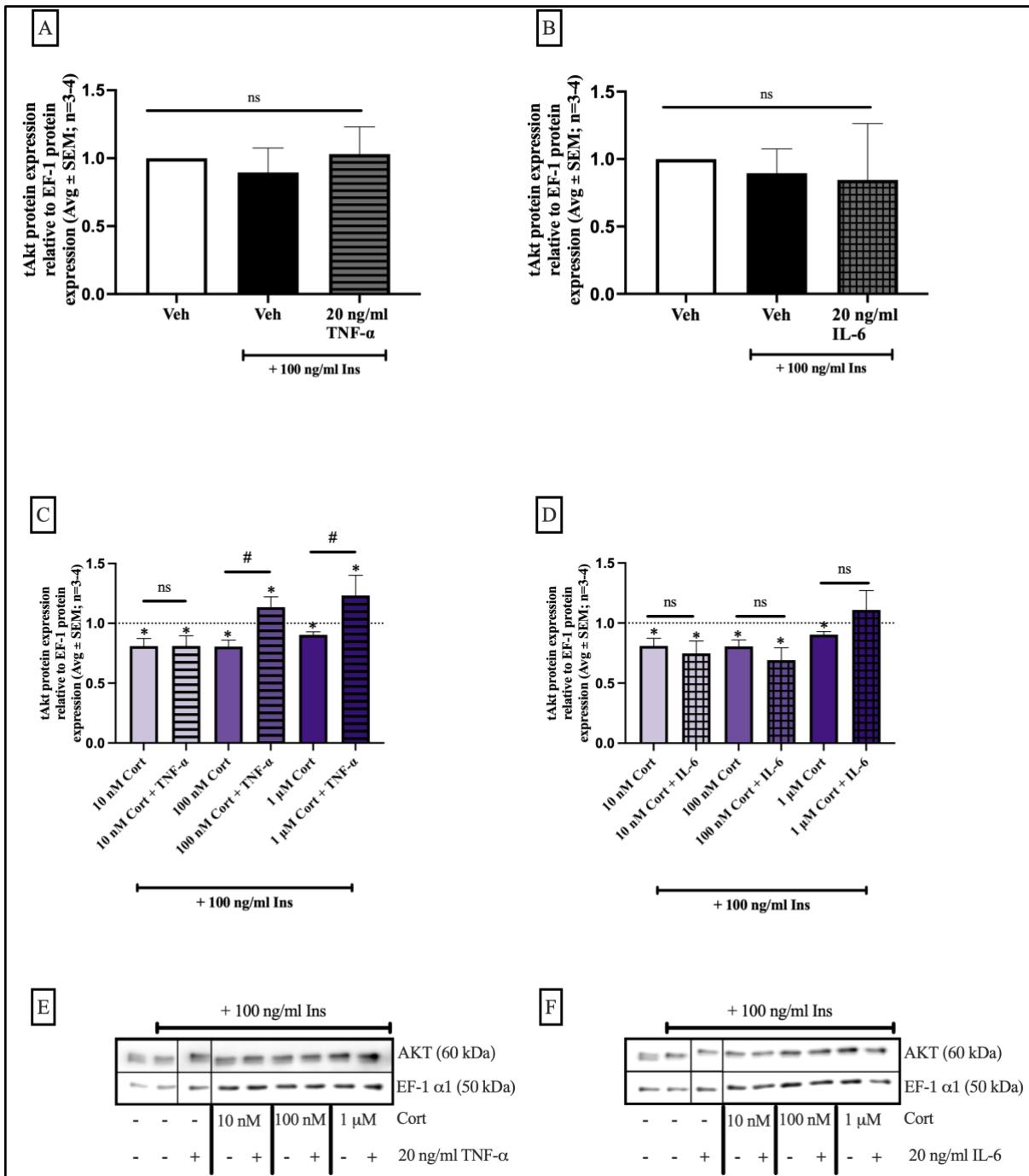


Figure 3.2. Corticosterone at high concentrations in the presence of TNF- α increases total AKT expression in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M corticosterone (Cort), or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells were treated with a combination of corticosterone 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Total AKT expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

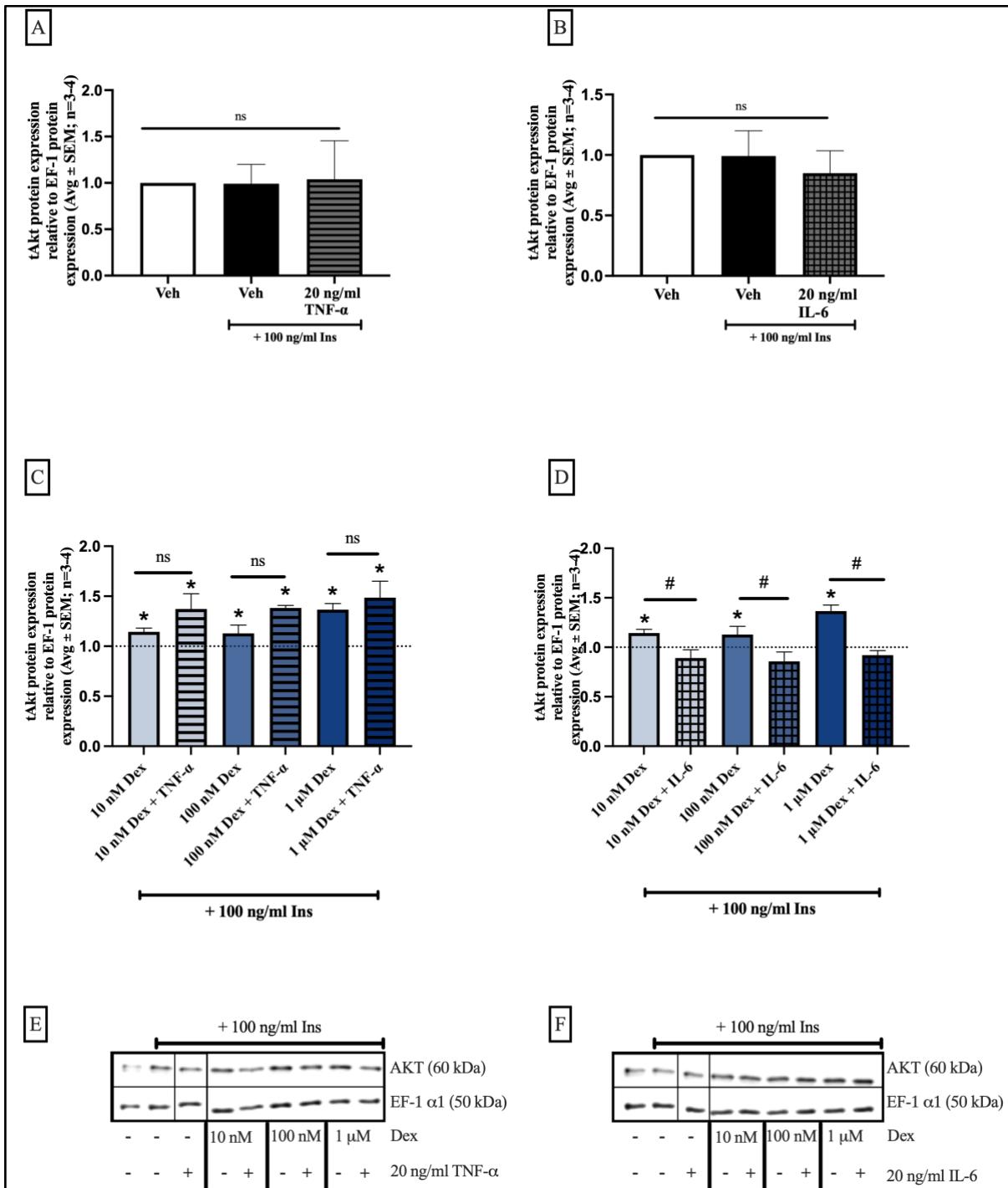


Figure 3.3. IL-6 antagonises dex-induced increase of total AKT in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dex, or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells were treated with a combination of dex at the various concentrations and either of the two cytokines. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Total AKT expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (A, C, E) and IL-6 (B, D, F). Statistical analysis was performed using an unpaired student's t-test with * ($p<0.05$) representing comparison to the insulin control only whilst # ($p<0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p>0.05$) represents no significance.

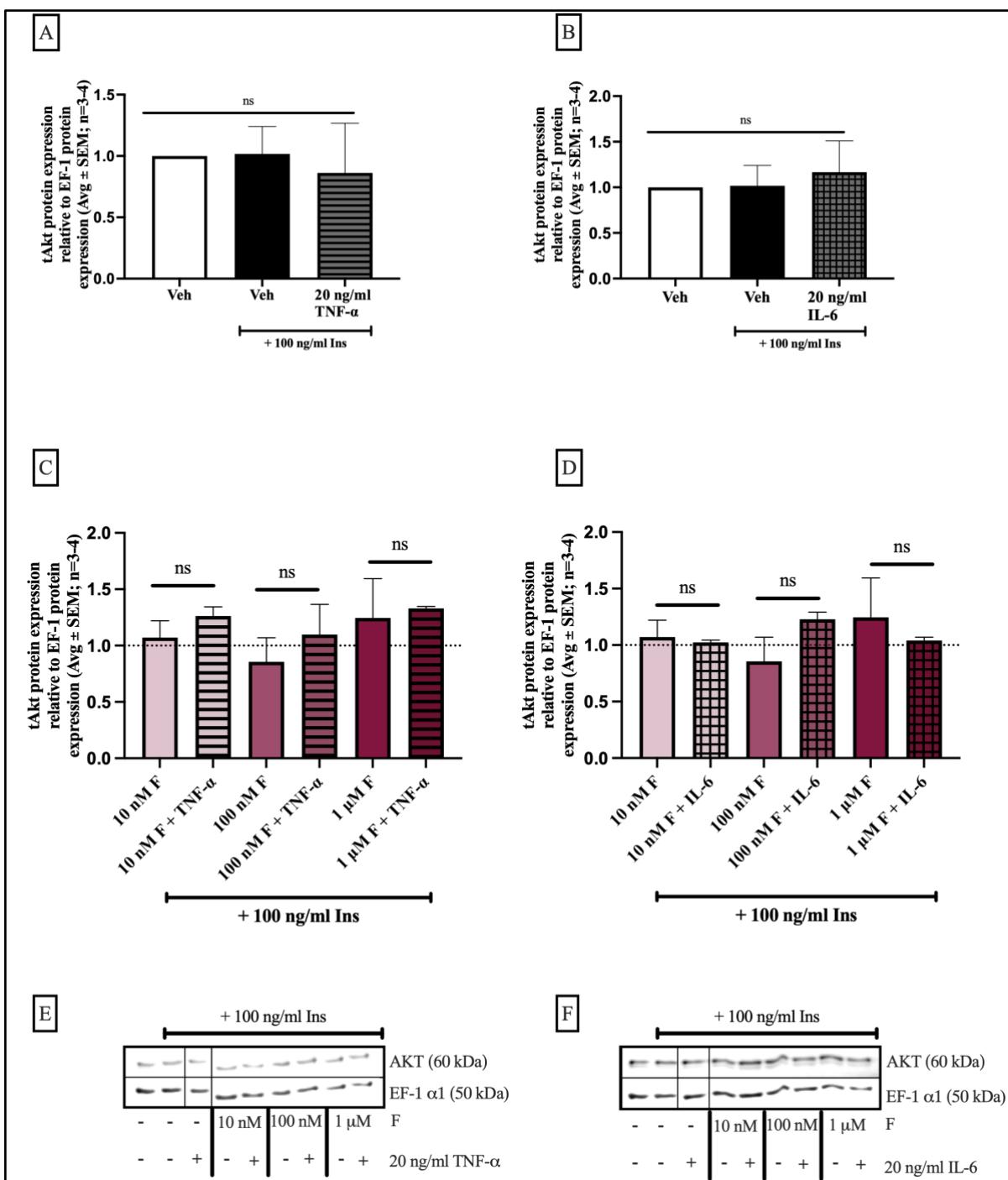


Figure 3.4. Cortisol in the absence or presence of TNF- α or IL-6 has no effect on total AKT expression levels in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M cortisol (F), or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells were treated with a combination of cortisol at the various concentrations and either of the two cytokines. This was done for 24 hours. Cells were subsequently treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Total AKT expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (A, C, E) and IL-6 (B, D, F). Statistical analysis was performed using an unpaired student's t-test with * ($p<0.05$) representing comparison to the insulin control only whilst # ($p<0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p>0.05$) represents no significance.

3.2 Effects of the test compounds on phosphorylation of AKT at threonine 308.

Stimulation of the insulin signalling pathway leads to the phosphorylation of two critical residues belonging to AKT, with threonine 308, in the activation or T-loop being the first residue phosphorylated (92). Phosphorylation of threonine 308 is crucial for the catalytic activity of AKT (278). Failure to phosphorylate the enzyme for whatever reason, results in the inhibition of insulin-induced activation of AKT (44, 279).

In the absence of insulin, no phosphorylation of AKT at threonine 308 was observed (Fig. 3.5E & F). Exposure to 100 ng/ml insulin for 30 minutes significantly ($p<0.05$) increased the phosphorylation of AKT at threonine 308 between 7- and 17-fold in the BWTG3 and HepG2 cell lines, respectively (Figs. 3.5A & B, 3.6A & B, 3.7A & B, 3.8A & B). The pro-inflammatory cytokines TNF- α and IL-6 had no effect on the insulin-induced phosphorylation of AKT at threonine 308 in both hepatocyte cell models (Figs. 3.5A & B, 3.6A & B, 3.7A & B, 3.8A & B).

Treatment with dex in the absence and presence of the pro-inflammatory cytokines, TNF- α and IL-6, did not influence the ability of insulin to induce phosphorylation of AKT at threonine 308 in the BWTG3 cells (Fig. 3.5C - F). Similarly, corticosterone alone at the lower concentrations tested (10 nM and 100 nM) did not significantly ($p>0.05$) affect the insulin-induced phosphorylation of AKT at threonine 308. In contrast, 1 μ M corticosterone significantly ($p<0.05$) decreased insulin-induced phosphorylation of AKT at threonine 308 in BWTG3 cells (Fig. 3.6C & D). Corticosterone at concentrations 10 nM and 1 μ M in the presence of 20 ng/ml TNF- α significantly ($p<0.05$) increased the phosphorylation of AKT at threonine 308 in BWTG3 cells compared to corticosterone alone, although this was not significantly ($p>0.05$) different to the insulin only treatment (Fig. 3.6C). In contrast, 1 μ M corticosterone in the presence of IL-6 significantly ($p<0.05$) augmented the insulin-induced phosphorylation of AKT at threonine 308. IL-6 co-treated with the lower concentrations of corticosterone, 10 nM and 100 nM, however, did not affect insulin-induced phosphorylation of AKT at threonine 308 (Fig. 3.6D).

In contrast to the BWTG3 cells, in the HepG2 cell line, dex at all concentrations tested significantly ($p<0.05$) increased the insulin-induced phosphorylation, which was unaffected by the pro-inflammatory cytokines, TNF- α and IL-6 (Fig. 3.7C-F). Unlike dex, the endogenous GC, cortisol, in the absence and presence of TNF- α did not affect the insulin-induced

phosphorylation of AKT at threonine 308 (Fig. 3.8C & E). Similarly, IL-6 in the presence of cortisol had no significant ($p>0.05$) effect on the insulin-induced phosphorylation of AKT at threonine 308 (Fig. 3.8.D & F) except for 100 nM cortisol where the presence of IL-6 caused a decrease in phosphorylation. A significant ($p<0.05$) decrease in the insulin-induced phosphorylation of AKT at threonine 308 was observed when the HepG2 cells were treated with 100 nM cortisol in the presence of 20 ng/ml IL-6 compared to 100 nM cortisol treatment alone. This decrease in phosphorylation of AKT at threonine 308 when HepG2 cells were co-treated with 100 nM cortisol and IL-6 was also significantly ($p<0.05$) different to the insulin only treatment (Fig. 3.8.D).

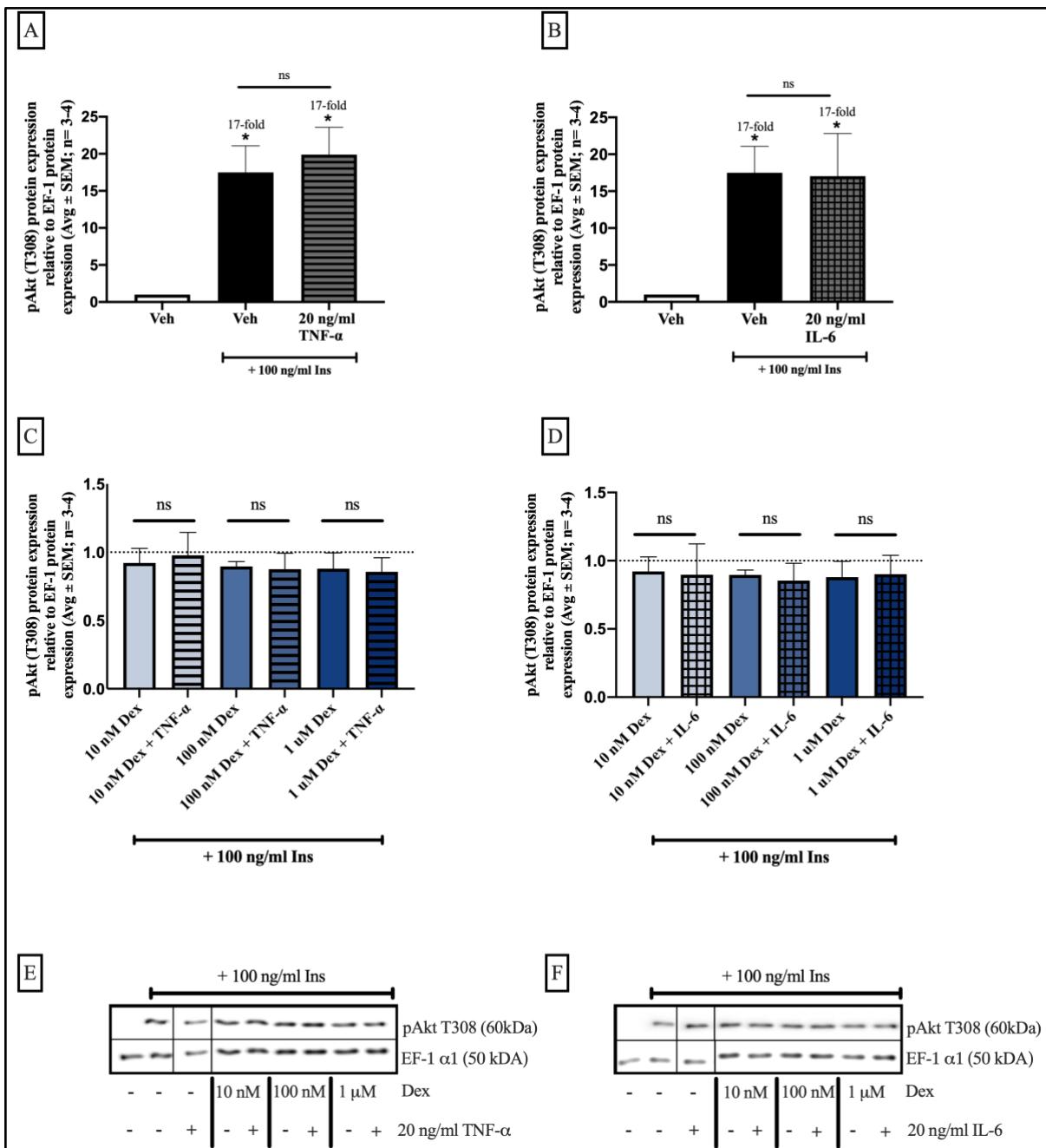


Figure 3.5 Dex in the absence and presence of TNF- α or IL-6 does not affect the insulin-induced phosphorylation of AKT at threonine 308 (T308) in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dex, or 20 ng/ml of either TNF- α 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Phospho-Akt (T308) expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (A, C, E) and IL-6 (B, D, F). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control; ns ($p > 0.05$) represents no significance.

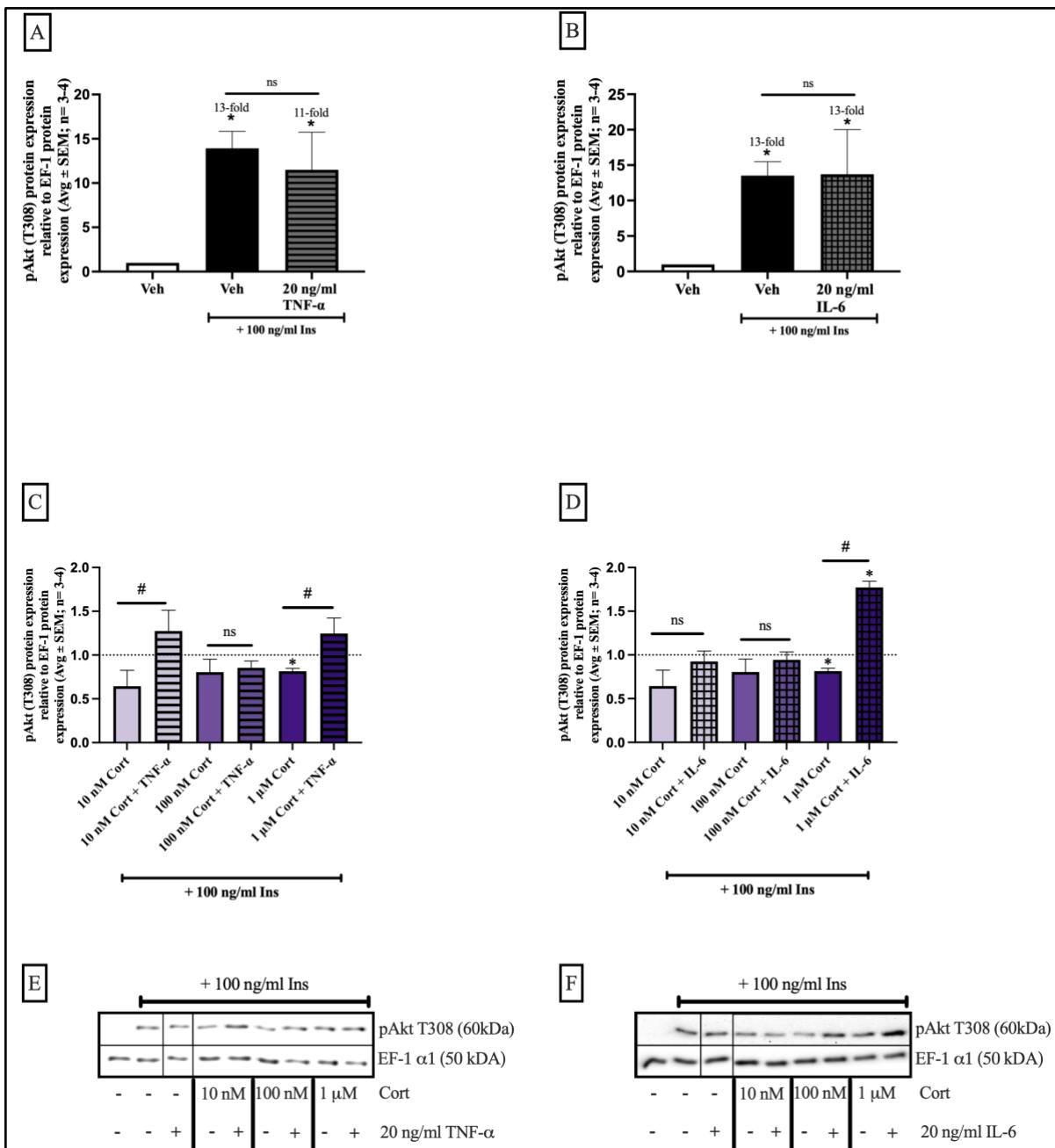


Figure 3.6 IL-6 co-operatively increases the insulin-induced phosphorylation of AKT at threonine 308 (T308) seen at high concentration of corticosterone tested in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M corticosterone (Cort), or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells were treated with a combination of corticosterone 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. Phospho-Akt (T308) expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

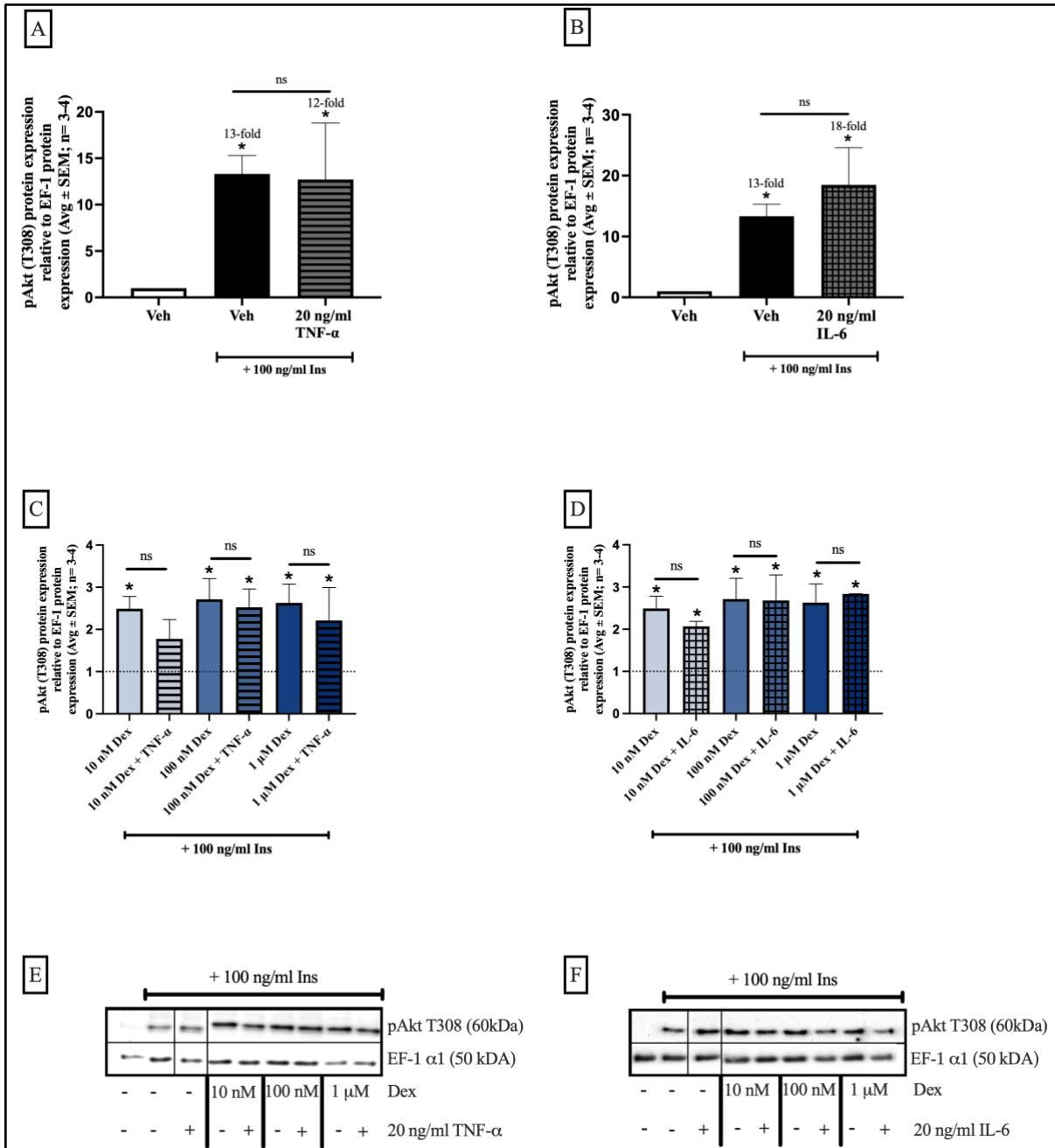


Figure 3.7 Treatment with dex in the absence and presence of TNF- α or IL-6 significantly increase the insulin-induced phosphorylation of AKT at threonine 308 (T308) in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dex, or 20 ng/ml 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Phospho-Akt (T308) expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control; ns ($p > 0.05$) represents no significance.

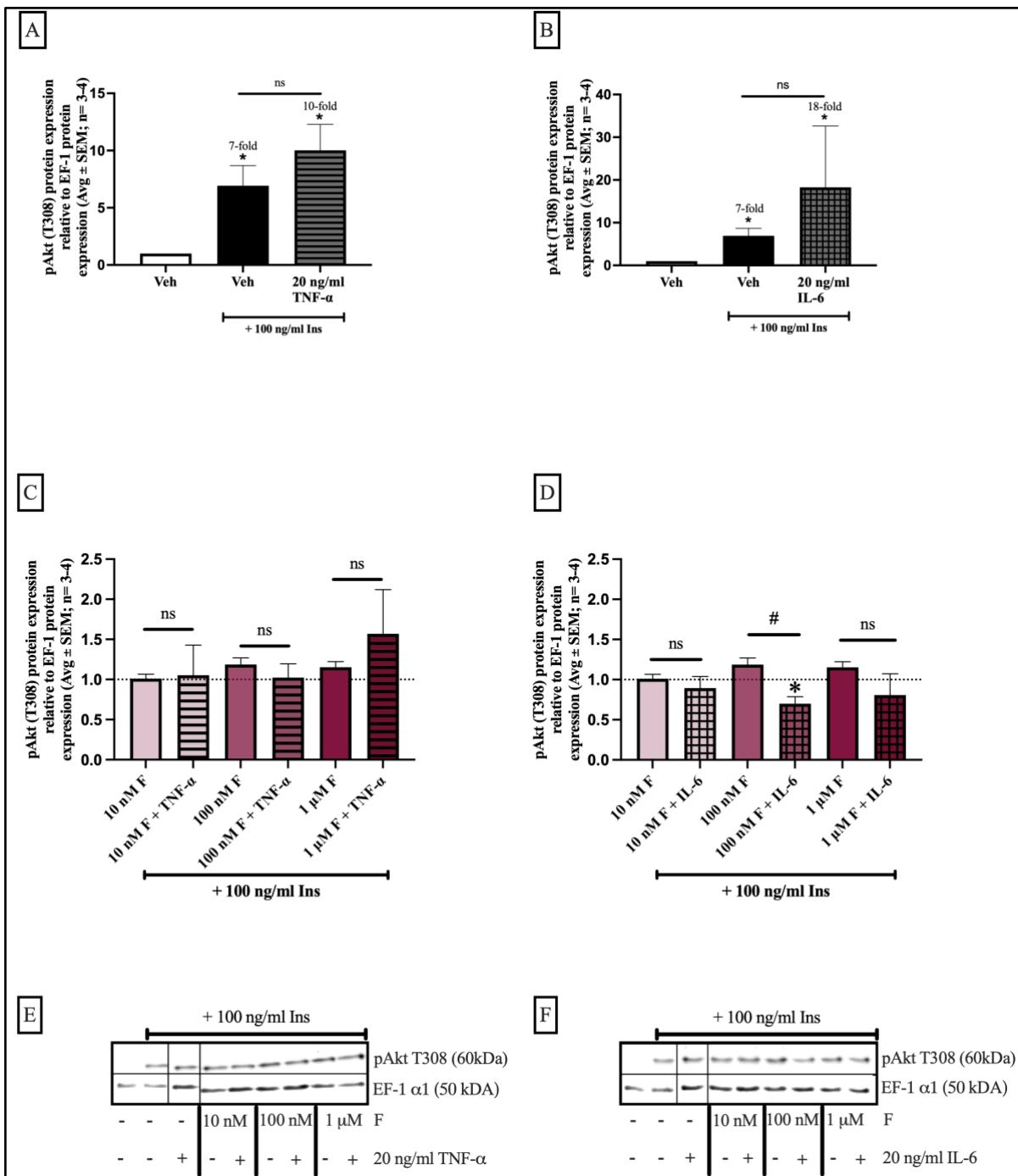


Figure 3.8 Cortisol only at 100 nM in the presence of IL-6 decreased insulin-induced phosphorylation of AKT at threonine 308 (T308) in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M cortisol (F), or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells were treated with a combination of cortisol 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Phospho-Akt (T308) expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (A, C, E) and IL-6 (B, D, F). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p<0.05$) representing comparison to the insulin control only whilst # ($p<0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p>0.05$) represents no significance.

3.3 Effects of the test compounds on phosphorylation of AKT at serine 473

The second critical residue of AKT to be phosphorylated is serine 473 situated in the hydrophobic domain of the enzyme (278). Phosphorylation of this residue further augments the enzymatic activity of AKT and expands its substrate scope (278). Due to the limit of detection little to no phosphorylation of AKT at serine 473 was observed in the absence of insulin in both hepatocyte cell models (Figs. 3.9A & B, 3.10A & B, 3.11A & B, 3.12A & B), similar to what was observed with threonine 308 AKT phosphorylation (Figs. 3.5A & B, 3.6A & B, 3.7A & B, 3.8A & B). Treatment with 100 ng/ml insulin resulted in a significant ($p<0.05$) increase of phosphorylated AKT at serine 473 in both cell lines used (Fig. 3.9A & B, 3.10A & B, 3.11A & B, 3.12A & B). TNF- α and IL-6, like with threonine 308 AKT phosphorylation, had no significant ($p>0.05$) effect on insulin-induced phosphorylation of AKT at serine 473 (Fig. 3.9A & B, 3.10A & B, 3.11A & B, 3.12A & B).

Dex treatment alone did not affect the insulin-induced phosphorylation of AKT at serine 473 (Fig. 3.9C & D) in the murine BWTG3 cells. However, in the presence of TNF- α , 10 nM and 100 nM dex significantly ($p<0.05$) increased the insulin-induced AKT phosphorylation at serine 473. This increase in serine 473 phosphorylation of AKT however, is absent when TNF- α is co-treated with the highest concentration of dex (1 μ M) used (Fig. 3.9.C). In contrast, only 100 nM dex in the presence of IL-6 significantly ($p<0.05$) increased insulin-induced serine 473 phosphorylation of AKT compared to insulin in the absence and presence of 100 nM dex. Co-treatment with IL-6 and 10 nM or 1 μ M dex had no significant ($p>0.05$) effect on the phosphorylation status of AKT at serine 473 compared to insulin alone or insulin in the presence of dex only (Fig. 3.9D). Corticosterone treatment had no significant ($p>0.05$) effect on insulin-induced serine 473 phosphorylation of AKT except for 1 μ M corticosterone in BWTG3 cells (Fig. 3.10C & D). Although 100 nM corticosterone did not affect the insulin-induced phosphorylation of AKT at serine 473, in the presence of 20 ng/ml TNF- α insulin-induced phosphorylation of this serine residue significantly ($p<0.05$) increased in BWTG3 cells. Furthermore, the significant ($p<0.05$) increase in insulin-induced AKT phosphorylation by 1 μ M corticosterone is potentiated by co-treating with TNF- α (Fig. 3.10C & E). In contrast, the increase in insulin-induced serine 473 phosphorylation of AKT in BWTG3 cells by 1 μ M corticosterone was significantly ($p<0.05$) attenuated when co-treated with IL-6. This decrease in phosphorylation, however, is not significantly ($p>0.05$) different from the insulin only

treatment. In addition, IL-6 had no significant ($p>0.05$) effect on lower concentrations (10 nM and 100nM) of corticosterone treatment Fig. 3.10D & F).

In the HepG2 cells, similar to the BWTG3 cells, dex treatment augmented insulin-induced phosphorylation of AKT at serine 473 (Fig. 3.11C & D). This increased response, however, was significantly ($p<0.05$) inhibited by TNF- α when co-treated with 100 nM dex, although the response remained significantly different from the insulin-only induced AKT phosphorylation at serine 473. Furthermore, although significance could not be established, TNF- α was also able to inhibit 10 nM dex to an extent similar to the insulin only treatment. In contrast, TNF- α significantly ($p<0.05$) enhanced the effect of the highest concentration (1 μ M) dex tested on insulin-induced phosphorylation of AKT at serine 473 (Fig. 3.11C & E). Similarly, IL-6 in the presence of 1 μ M dex significantly ($p<0.05$) increased insulin-induced phosphorylation of AKT at serine 473 compared to 1 μ M dex treatment. In contrast, IL-6 in the presence of the lower dex concentrations (10 nM and 100 nM), significantly ($p<0.05$) attenuated the increase in insulin-induced serine 473 phosphorylation of AKT mediated by dex, however, only IL-6 co-treated with 10 nM dex resulted in a phosphorylation status of AKT at serine 473 similar to the insulin treatment alone (Fig. 3.11D & F). In contrast to the synthetic GC treatment studies, insulin induced AKT phosphorylation at serine 473 was only significantly ($p<0.05$) affected by the highest concentration (1 μ M) of cortisol investigated (Fig. 3.12). The effect of 1 μ M cortisol on insulin-induced phosphorylation of AKT at serine 473 is significantly ($p<0.05$) inhibited by TNF- α to similar levels of that induced by insulin alone (Fig. 3.12C & E). Unlike TNF- α , co-treatment with IL-6 did not influence the effect of cortisol on insulin-induced AKT serine 473 phosphorylation (Fig. 3.12D & F).

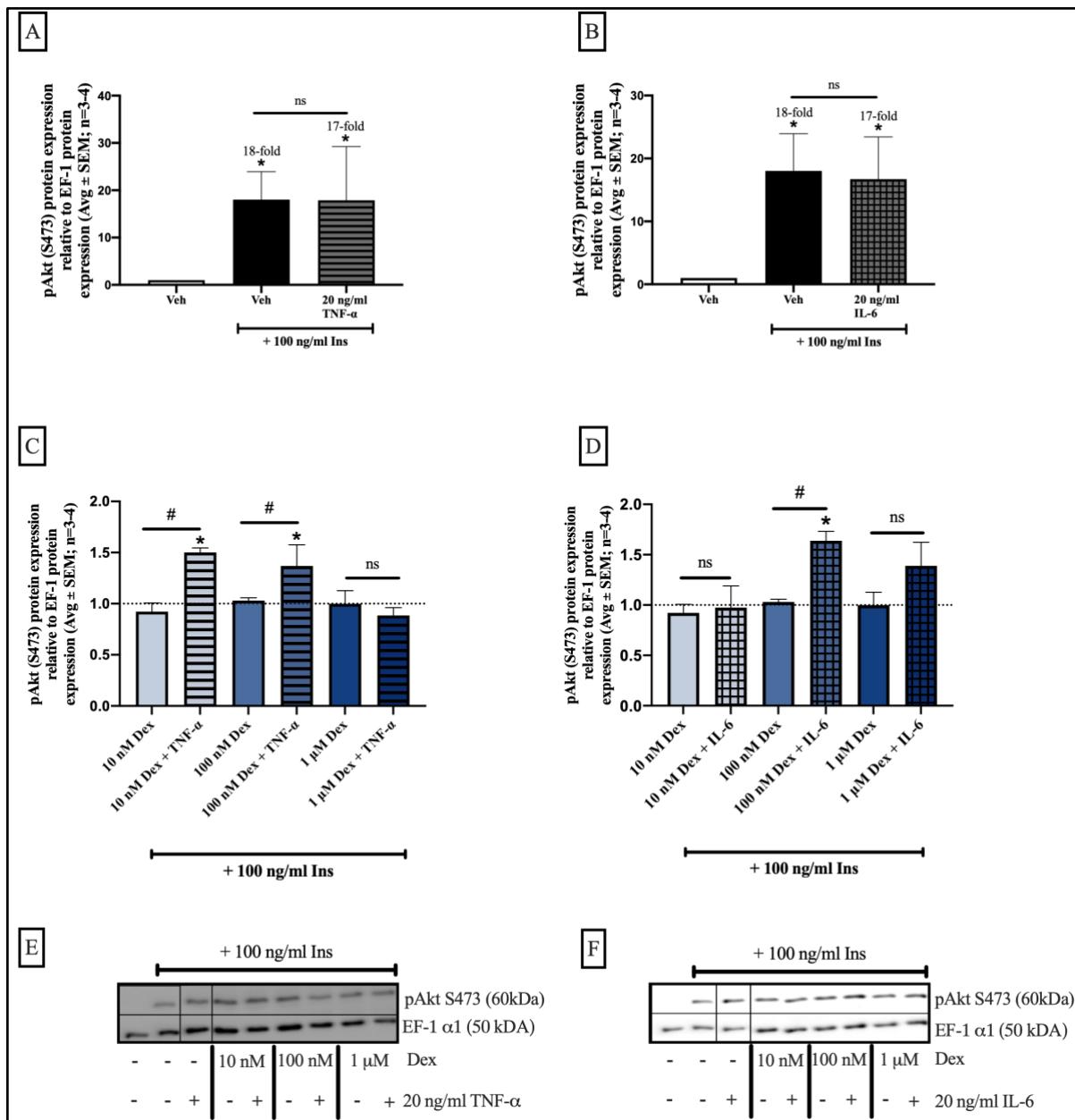


Figure 3.9 Dex and the cytokines exhibit co-operative effects on insulin-induced phosphorylation of AKT at serine 473 in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dexamethasone (Dex), or 20 ng/ml of either TNF- α or IL-6. For 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Phospho-Akt (S473) expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (A, C, E) and IL-6 (B, D, F). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p<0.05$) representing comparison to the insulin control only whilst # ($p<0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p>0.05$) represents no significance.

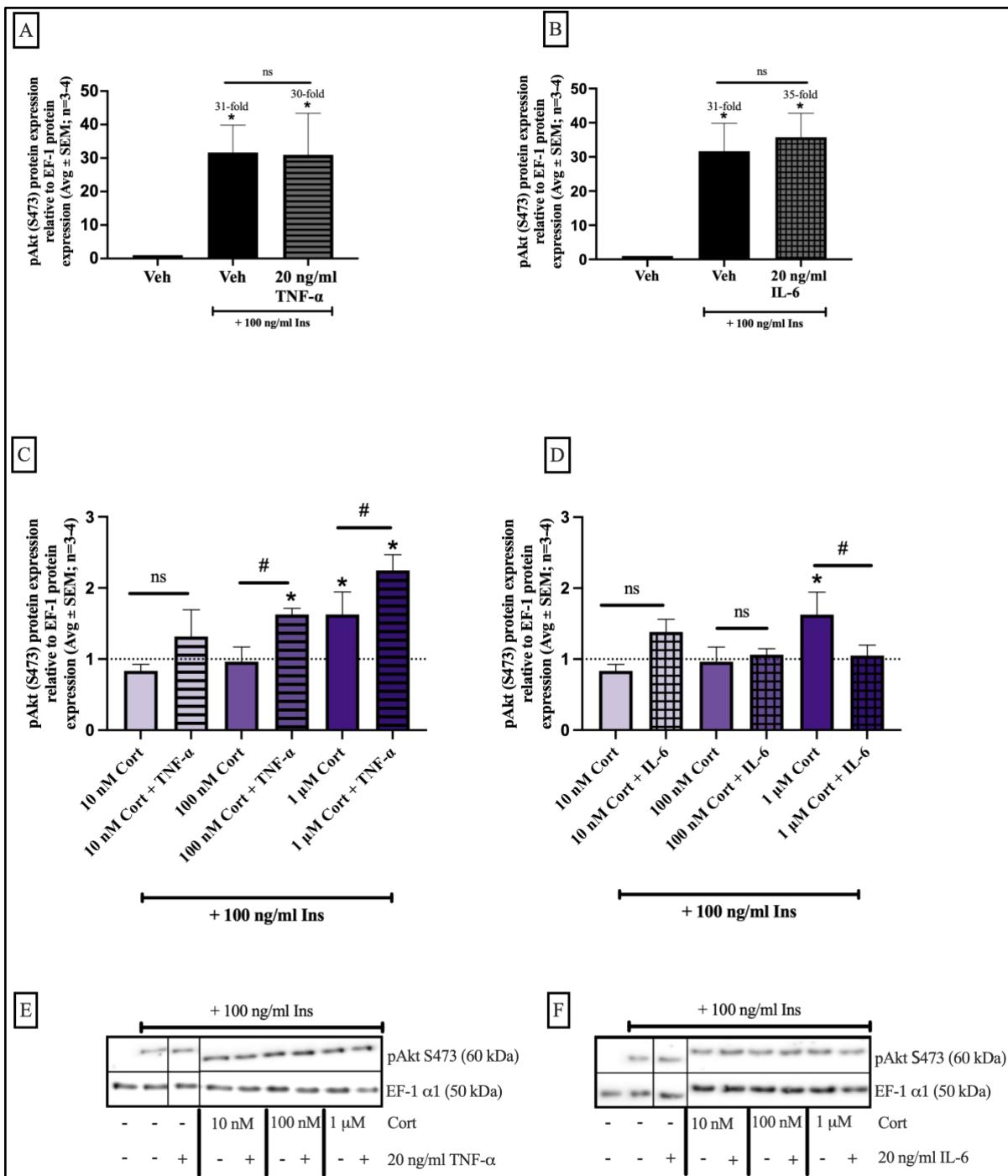


Figure 3.10 TNF- α and IL-6 exhibit different effects in the presence of corticosterone on insulin-induced phosphorylation of AKT at serine 473 in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M corticosterone (Cort), or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells were treated with a combination of corticosterone 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Phospho-Akt (S473) expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (A, C, E) and IL-6 (B, D, F). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p<0.05$) representing comparison to the insulin control only whilst # ($p<0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p>0.05$) represents no significance.

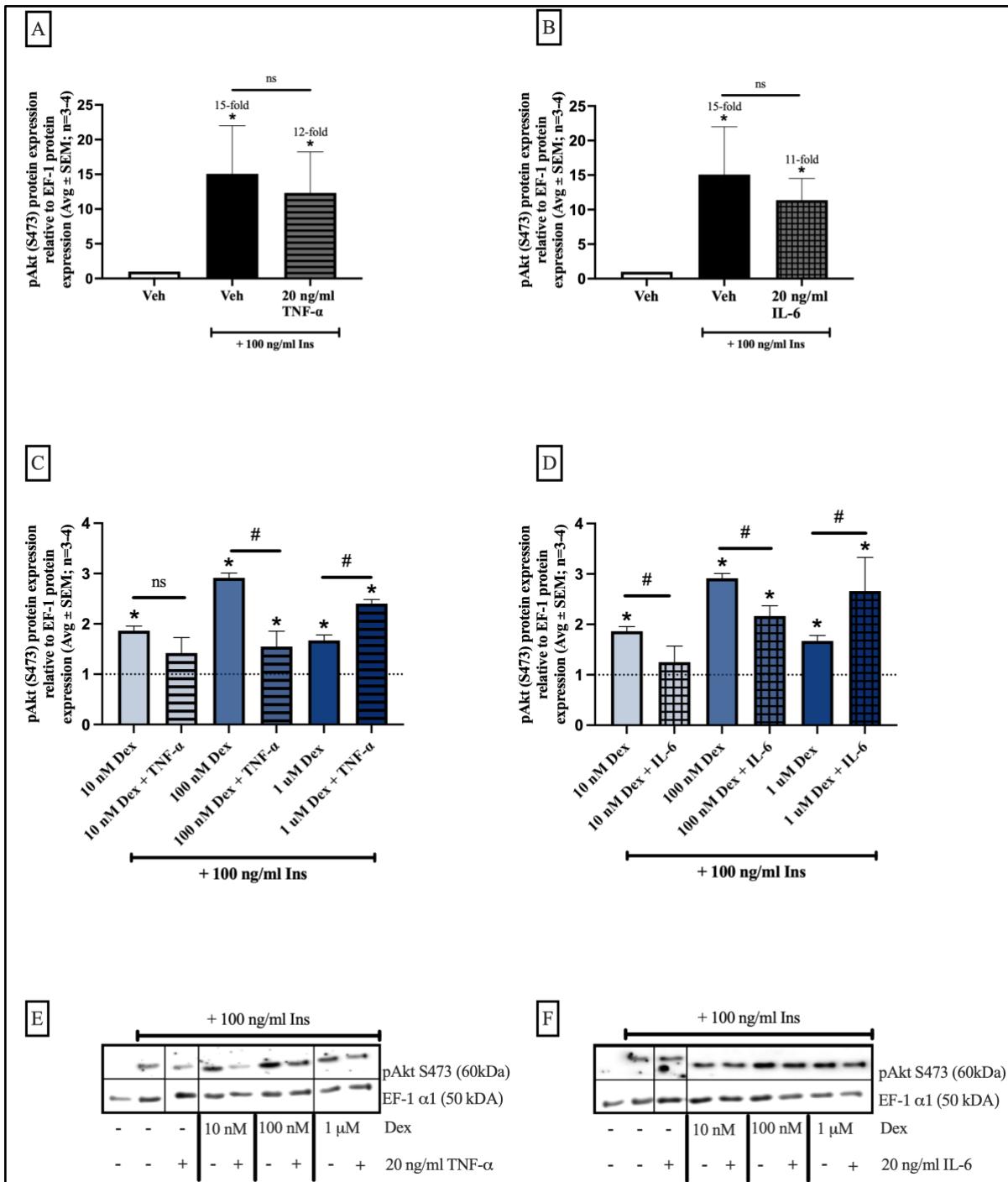


Figure 3.11 TNF- α and IL-6 in the presence of dex exhibit similar effects on insulin-induced phosphorylation of AKT at serine 473 (S473) in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dex, or 20 ng/ml of either TNF- α or IL-6. 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Phospho-Akt (S473) expression was measured and quantified using EF-1 α as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

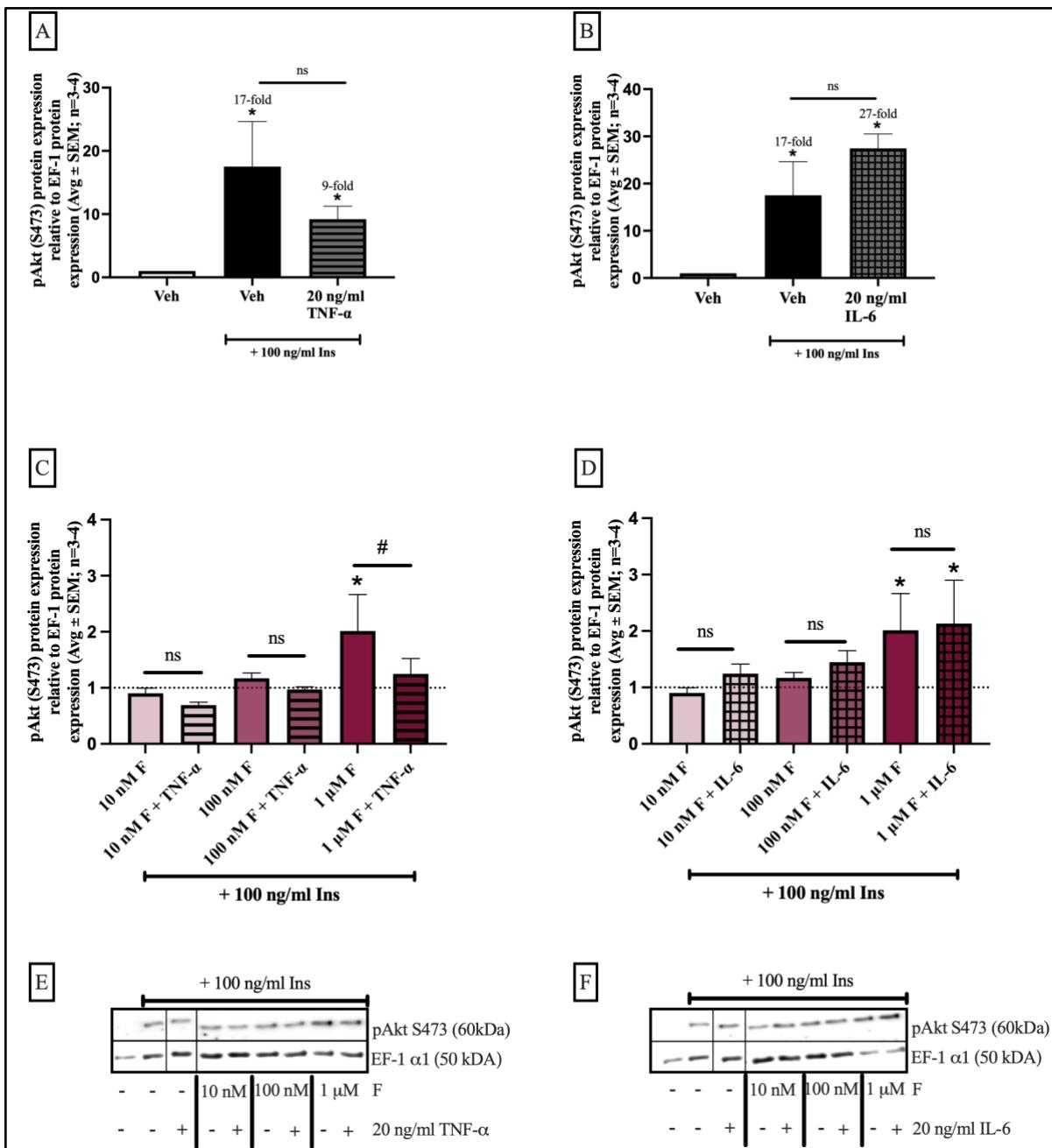


Figure 3.12 TNF- α alone antagonises the insulin-induced phosphorylation of AKT at serine 473 (S473) seen at high concentrations of cortisol in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M cortisol (F), or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells were treated with a combination of cortisol 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Phospho-Akt (S473) expression was measured and quantified using EF-1 α 1 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents either three or four independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

3.4 Effects of the test compounds on GSK-3 protein expression

As described in chapter one (section 1.3.3) glycogen synthesis is regulated by GSK-3. In resting cells, GSK-3 phosphorylates GS, thereby inactivating it, which results in the inhibition of glycogen synthesis (50, 121, 280). Phosphorylation of GSK-3 α and GSK-3 β at tyrosine 279 and tyrosine 216, respectively is required for maximal GSK-3 activity (51).

Treatment with 100 ng/ml insulin in the absence and presence of 20 ng/ml of either TNF- α or IL-6 did not affect the protein expression of either GSK-3 α (Fig. 3.13A & B) or GSK-3 β (Fig. 3.13C & D) in the BWTG3 cells.

In the absence and presence of either of the pro-inflammatory cytokines, neither dex nor corticosterone affected the protein expression of GSK-3 α in BWTG3 cells in comparison to the insulin only treatment (which was set to 1), except for the co-treatment of 1 μ M dex with IL-6 which decreased GSK-3 α protein levels, however, not significantly ($p>0.05$) compared to 1 μ M dex alone (Fig. 3.14A & B). In contrast, 1 μ M dex and corticosterone significantly ($p<0.05$) increased GSK-3 β protein levels in BWTG3 cells that was unaffected by co-treating with 20 ng/ml TNF- α (Fig. 3.14C). Unlike co-treatment with TNF- α , IL-6 attenuated the increase in GSK-3 β protein levels induced by both GCs in BWTG3 cells although significance could only be established for the co-treatment with the endogenous GC, corticosterone. Specifically, corticosterone-induced GSK- β protein expression was significantly ($p<0.05$) attenuated by 20 ng/ml IL-6 to an extent significantly ($p<0.05$) lower than basal levels (insulin only treatment) (Fig. 3.14D).

In the HepG2 cell line, like with the BWTG3 cells, 100 ng/ml insulin treatment in the absence and presence of 20 ng/ml of either TNF- α or IL-6 had no effect on GSK-3 α (Fig. 3.15A & B) or GSK-3 β (Fig. 3.15C & D) protein expression. Similarly, GSK-3 α protein expression was not affected by either dex or cortisol in the absence and presence of either of the pro-inflammatory cytokines (Fig. 3.16A & B). Whilst both the exogenous and endogenous GCs affected GSK-3 β protein expression in the mouse hepatocyte cell line, in the HepG2 cells only the endogenous GC, cortisol, significantly ($p<0.05$) increased GSK-3 β protein expression (Fig. 3.16C & D). Like with the BWTG3 cell line, only IL-6, and not TNF- α , was able to attenuate the cortisol-induced increase in GSK-3 β protein expression (Fig. 3.16C).

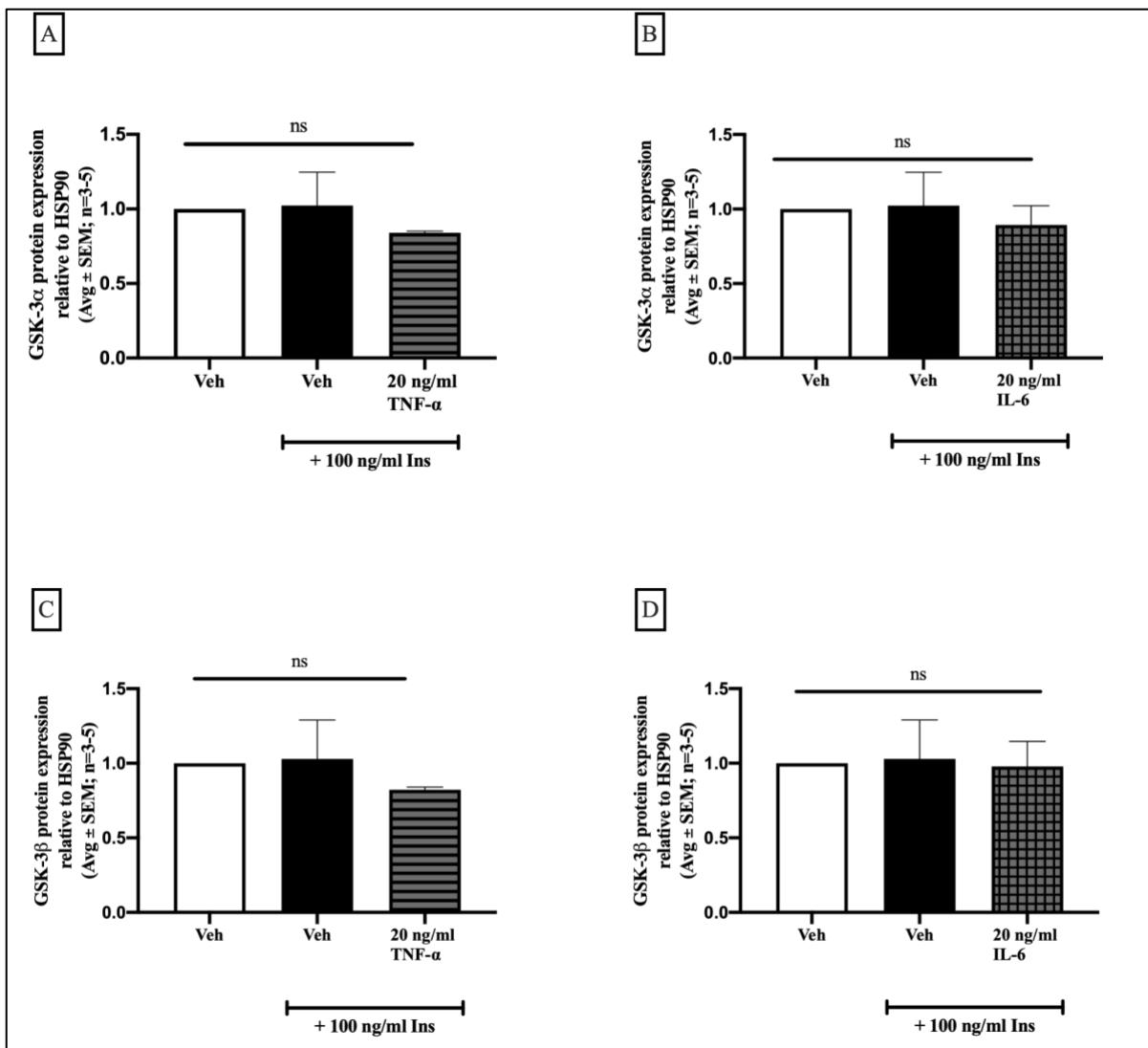


Figure 3.13. Neither insulin nor the cytokines affect GSK-3 protein expression in the BWTG3 cell line. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. GSK-3 α (**A, B**) and GSK-3 β (**C, D**) expression was measured and quantified using HSP90 as the loading control. Images are separated by cytokine type, i.e TNF- α (**A, C**) and IL-6 (**B, D**). Data shown represents between three and five independent experiments. Statistical analysis comparing the treatment conditions to one another was performed using an unpaired student's t-test; ns (p>0.05) represents no significance.

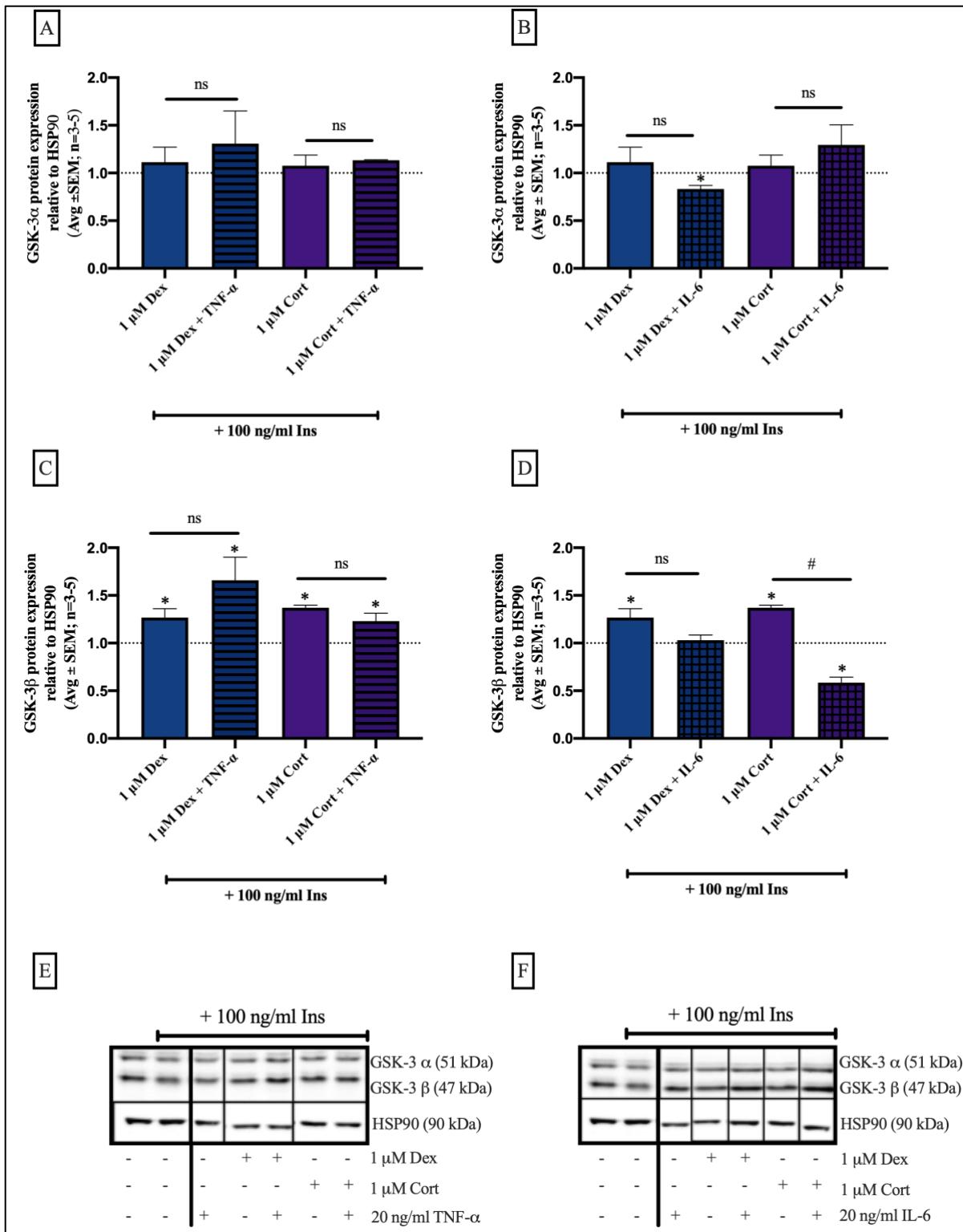


Figure 3.14. IL-6 antagonised the corticosterone-induced increase in GSK-3 β in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 1 μ M dex or corticosterone (cort), in the absence or presence of 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. GSK-3 α (**A, B**) and GSK-3 β (**C, D**) expression was measured and quantified using HSP90 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents between three and five independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

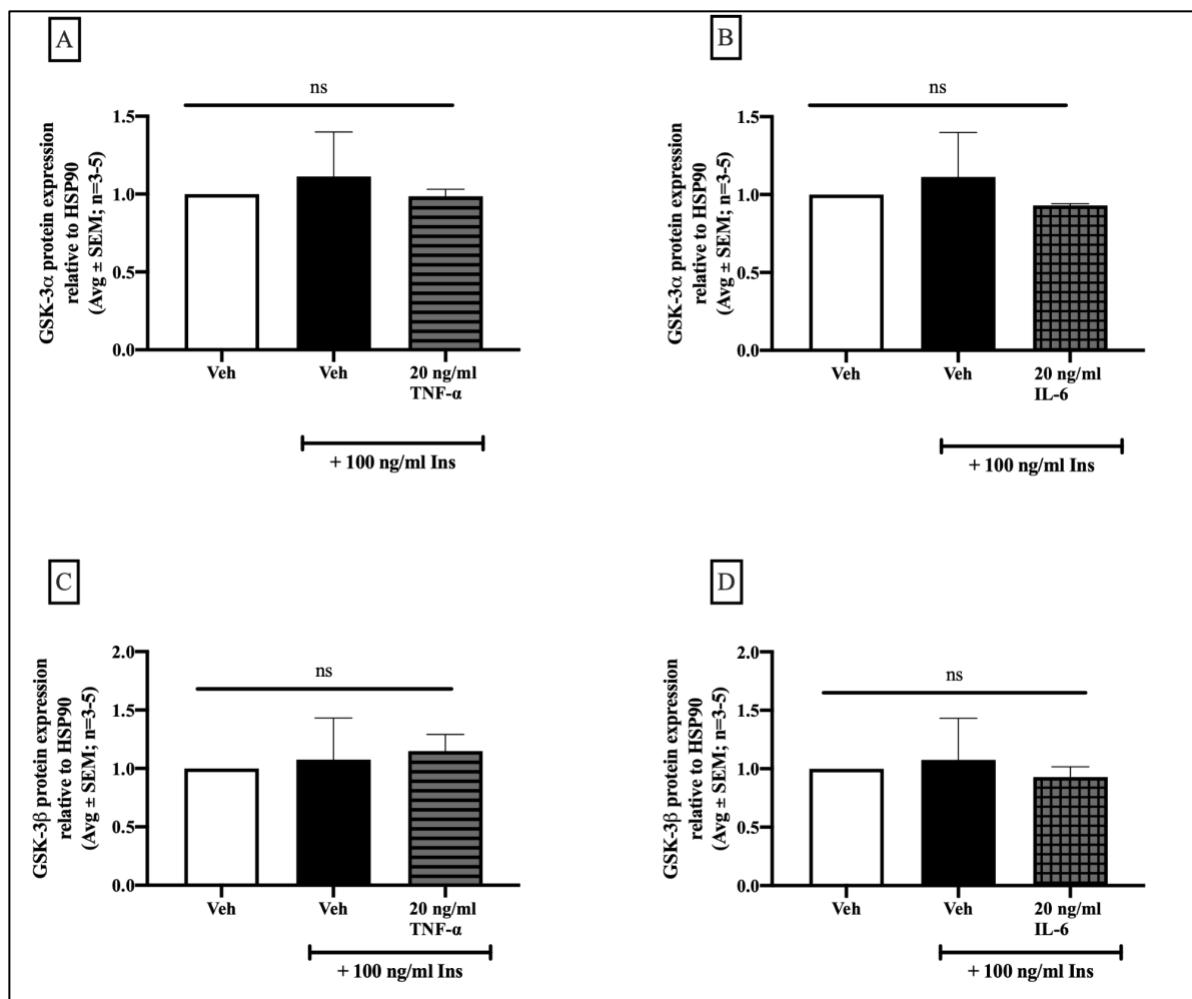


Figure 3.15. Neither insulin nor the cytokines affect the protein expression of GSK-3 in the HepG2 cell line. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. GSK-3 α (A, B) and GSK-3 β (C, D) expression was measured and quantified using HSP90 as the loading control. Images are separated by cytokine type, i.e TNF- α (A, C) and IL-6 (B, D). Data shown represents between three and five independent experiments. Statistical analysis comparing the treatment conditions to one another was performed using an unpaired student's t-test; ns represents no significance.

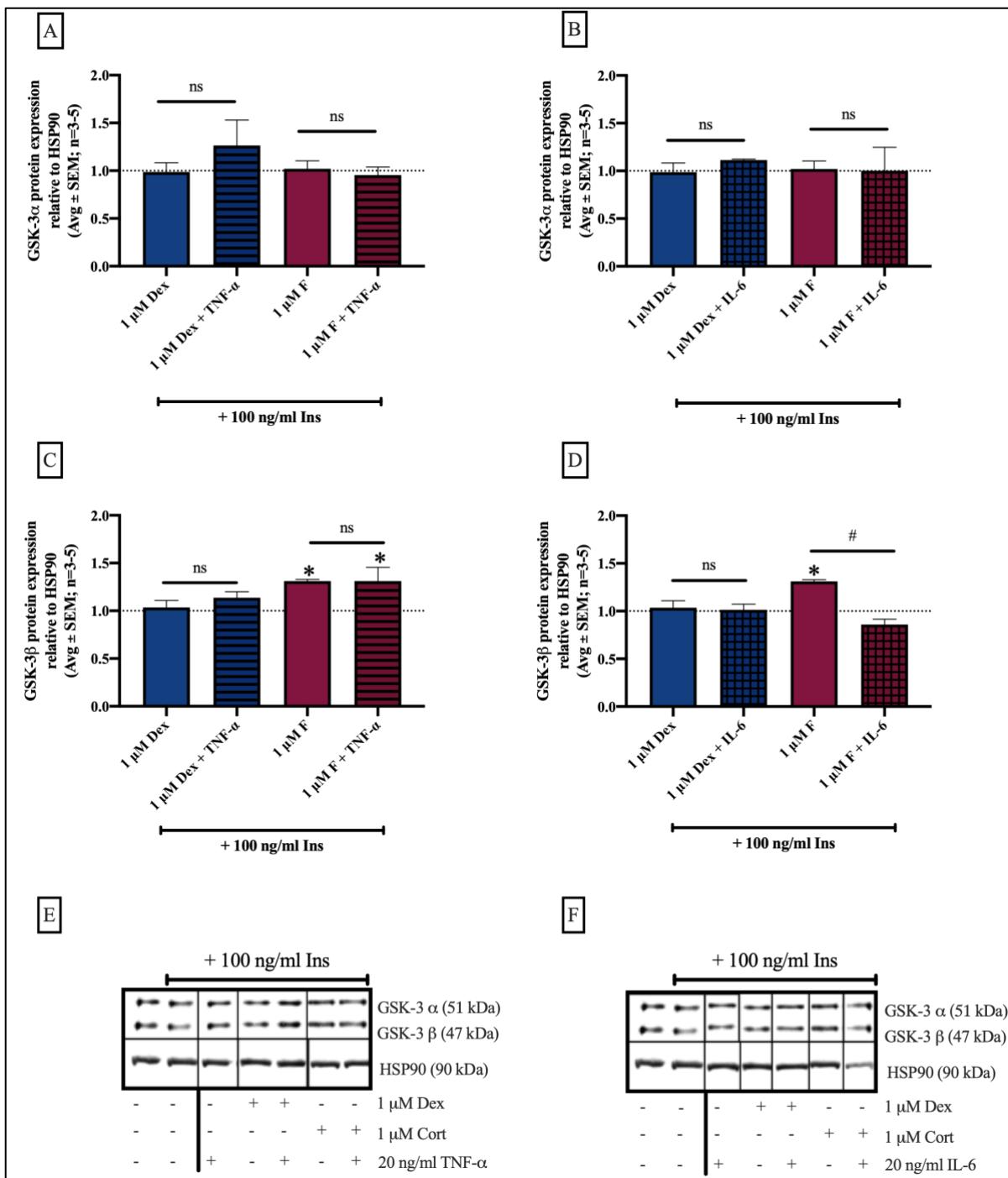


Figure 3.16. IL-6 but not TNF- α antagonises the cortisol-induced increase in GSK-3 β expression in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 1 μ M dexamethasone (dex) or cortisol (F), in the absence or presence of 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. GSK-3 α (**A, B**) and GSK-3 β (**C, D**) expression was measured and quantified using HSP90 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e. TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents between three and five independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

3.5 Effects of the test compounds on the tyrosine phosphorylation of GSK-3

In resting cells, GSK-3 is basally active and highly phosphorylated at key tyrosine residues (Tyr 279 on GSK-3 α and Tyr 216 on GSK-3 β) (51, 281, 282). Phosphorylation of GSK-3 at these residues increase the kinase activity of the enzyme, without which the kinase activity of GSK-3 would be impaired (49).

Insulin (100 ng/ml) in the absence and presence of 20 ng/ml of either TNF- α or IL-6 had no effect on the phosphorylation of either GSK-3 α at tyrosine 279 (Fig. 3.17A & B) or GSK-3 β at tyrosine 216 (Fig. 3.17C & D) in BWTG3 cells. All treatment with the GCs and the pro-inflammatory cytokines were done in the presence of insulin with phosphorylation of tyrosine 279 of GSK-3 α or at tyrosine 216 of GSK-3 β in the presence of insulin alone set as 1 for analysis.

Dex in the absence and presence of 20 ng/ml of either of the pro-inflammatory cytokines did not affect tyrosine 279 phosphorylation of GSK-3 α (Fig 3.18A & B). In contrast, 1 μ M corticosterone significantly ($p<0.05$) decreased GSK-3 α phosphorylation at tyrosine 279 (Fig. 3.18A & B), which was then only attenuated significantly ($p<0.05$) by IL-6 (Fig. 3.18B), but not TNF- α (Fig. 3.18A), although it did increase GSK-3 α phosphorylation at tyrosine 279 to levels similar to that of the cells stimulated with insulin only i.e., basal levels.

Similar to GSK-3 α phosphorylation at tyrosine 279, 1 μ M dex alone had no significant ($p>0.05$) effect on GSK-3 β phosphorylation at tyrosine 216 (Fig. 3.18C & D). However, dex in the presence of 20 ng/ml TNF- α significantly ($p<0.05$) increased phosphorylation of GSK-3 β at tyrosine 216 when compared to insulin only treatment, although it was not statistically different from dex treatment alone (Fig. 3.18C). In contrast, 1 μ M dex in the presence of 20 ng/ml IL-6 significantly ($p<0.05$) decreased the tyrosine 216 phosphorylation of GSK-3 β compared to insulin in the absence and presence of dex (Fig. 3.18D). Unlike dex, 1 μ M corticosterone significantly ($p<0.05$) decreased the tyrosine 216 phosphorylation of GSK-3 β compared to insulin, which was inhibited by the pro-inflammatory cytokines although only significantly ($p<0.05$) for IL-6 (Fig. 3.18C & D). Although 20 ng/ml TNF- α was unable to significantly ($p>0.05$) inhibit corticosterone, it did restore phosphorylation of GSK-3 β at tyrosine 216 to basal levels and similar to cells treated with insulin only (Fig. 3.18C).

Similar to the mouse cell line, HepG2 cells treated with 100 ng/ml insulin in the absence or presence of 20 ng/ml TNF- α or IL-6 did not significantly ($p>0.05$) affect the phosphorylation of either GSK-3 α at tyrosine 279 (Fig. 3.19A & B) or GSK-3 β at tyrosine 216 (Fig. 3.19C & D).

Dex at a concentration of 1 μ M in the absence of the pro-inflammatory cytokines did not affect tyrosine 279 phosphorylation of GSK-3 α (Fig. 3.20A & B). However, dex co-treatment with either of the pro-inflammatory cytokines significantly ($p<0.05$) increased GSK-3 α phosphorylation at tyrosine 279 (Fig. 3.20A & B). Unlike dex, 1 μ M cortisol in the absence of the pro-inflammatory cytokines significantly ($p<0.05$) increased the tyrosine 279 phosphorylation of GSK-3 α compared to insulin only treatment. The addition of 20 ng/ml TNF- α potentiated the cortisol-induced increase in tyrosine 279 phosphorylation of GSK-3 α (Fig. 3.20A), whilst 20 ng/ml IL-6 had no effect on the ability of cortisol to increase tyrosine 279 phosphorylation of GSK-3 α . Thus, while both pro-inflammatory cytokines significantly ($p<0.05$) affected dex treatment, only TNF- α significantly ($p<0.05$) affected cortisol treatment (Fig. 3.20A & B).

Like with GSK-3 α phosphorylation at tyrosine 279, 1 μ M dex in the absence of the pro-inflammatory cytokines did not affect tyrosine 216 phosphorylation of GSK-3 β (Fig. 3.20C & D). Co-treatment with TNF- α or IL-6 however did result in a significant ($p<0.05$) increase in GSK-3 β phosphorylation at tyrosine 216, when compared to insulin only treatment as well as to dex only (Fig. 3.20C & D). Similar to the tyrosine 279 phosphorylation of GSK-3 α , 1 μ M cortisol in the absence of a pro-inflammatory cytokine increased tyrosine 216 phosphorylation of GSK-3 β when compared to insulin only treatment (Fig. 3.20C & D). In addition, like with GSK-3 α phosphorylation at tyrosine 279, TNF- α and not IL-6 was able to potentiate cortisol-induced GSK-3 β phosphorylation at tyrosine 216 (Fig. 3.20C & D), thus, again highlighting differential effects of the two pro-inflammatory cytokines when co-treated with the endogenous GC.

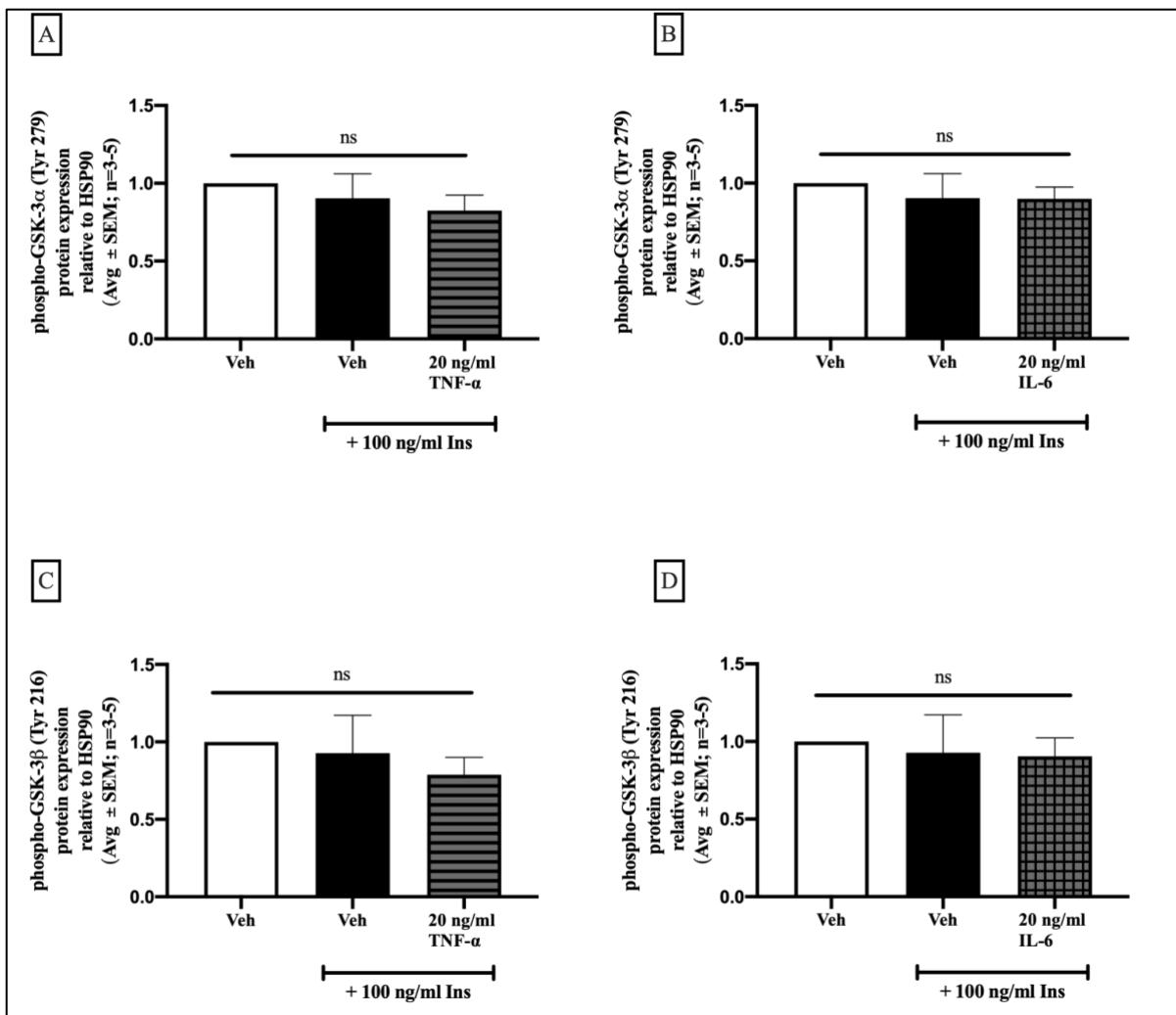


Figure 3.17. Neither insulin nor the cytokines affect the tyrosine phosphorylation of GSK-3 in the BWTG3 cell line. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Tyrosine phosphorylation of GSK-3 α (Tyr279) (A, B) and GSK-3 β (Tyr216) (C, D) was measured and quantified using HSP90 as the loading control. Images are separated by cytokine type, i.e TNF- α (A, C) and IL-6 (B, D). Data shown represents between three and five independent experiments. Statistical analysis comparing the treatment conditions to one another was performed using an unpaired student's t-test; ns (p>0.05) represents no significance.

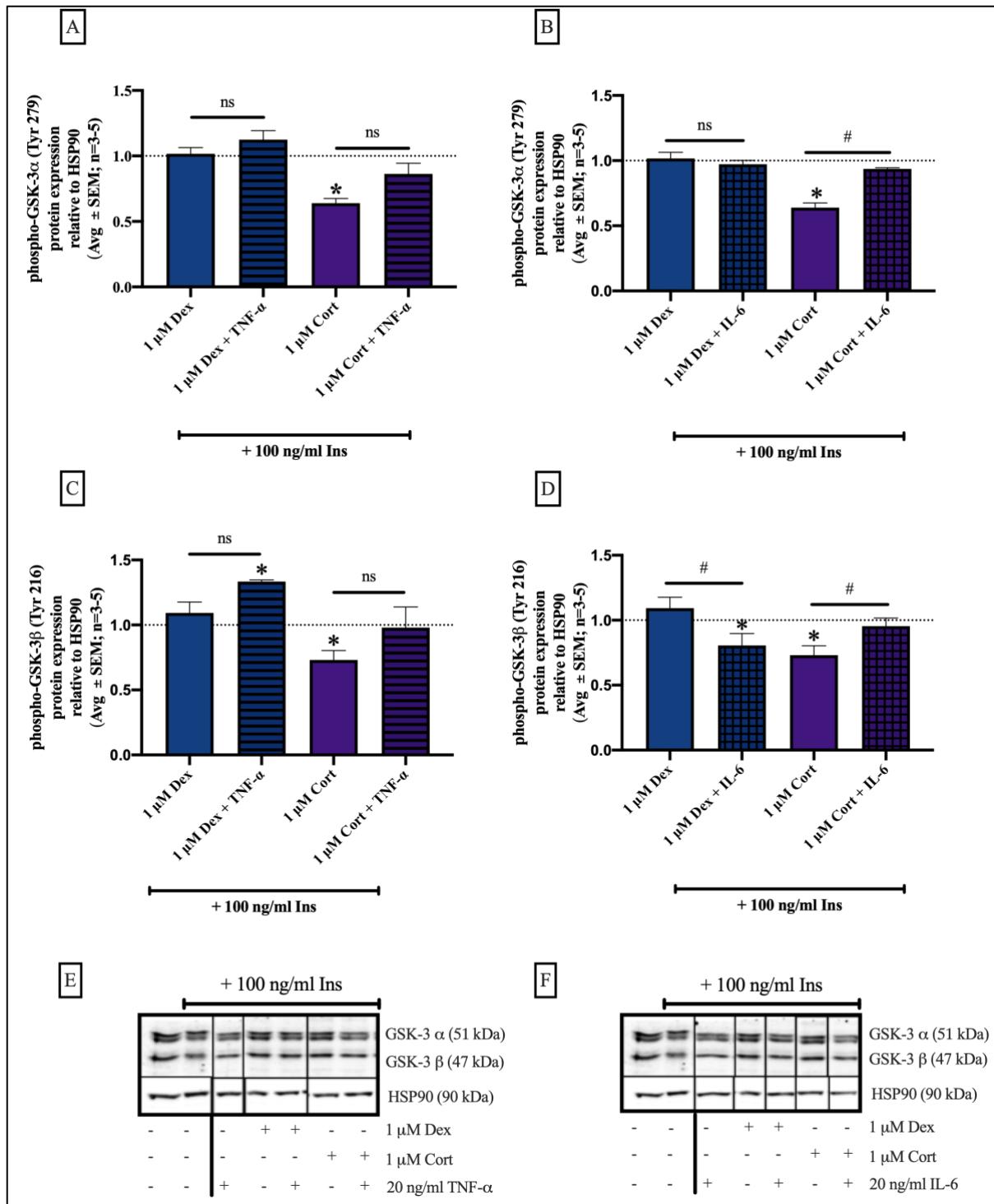


Figure 3.18. Corticosterone decreased GSK-3 α and GSK-3 β phosphorylation, with only IL-6 displaying antagonistic behaviour in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 1 μ M dexamethasone (dex) or corticosterone (cort), in the absence or presence of 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Tyrosine phosphorylation of GSK-3 α (Tyr279) (**A, B**) and GSK-3 β (Tyr216) (**C, D**) was measured and quantified using HSP90 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**A, C, E**) and IL-6 (**B, D, F**). Data shown represents between three and five independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

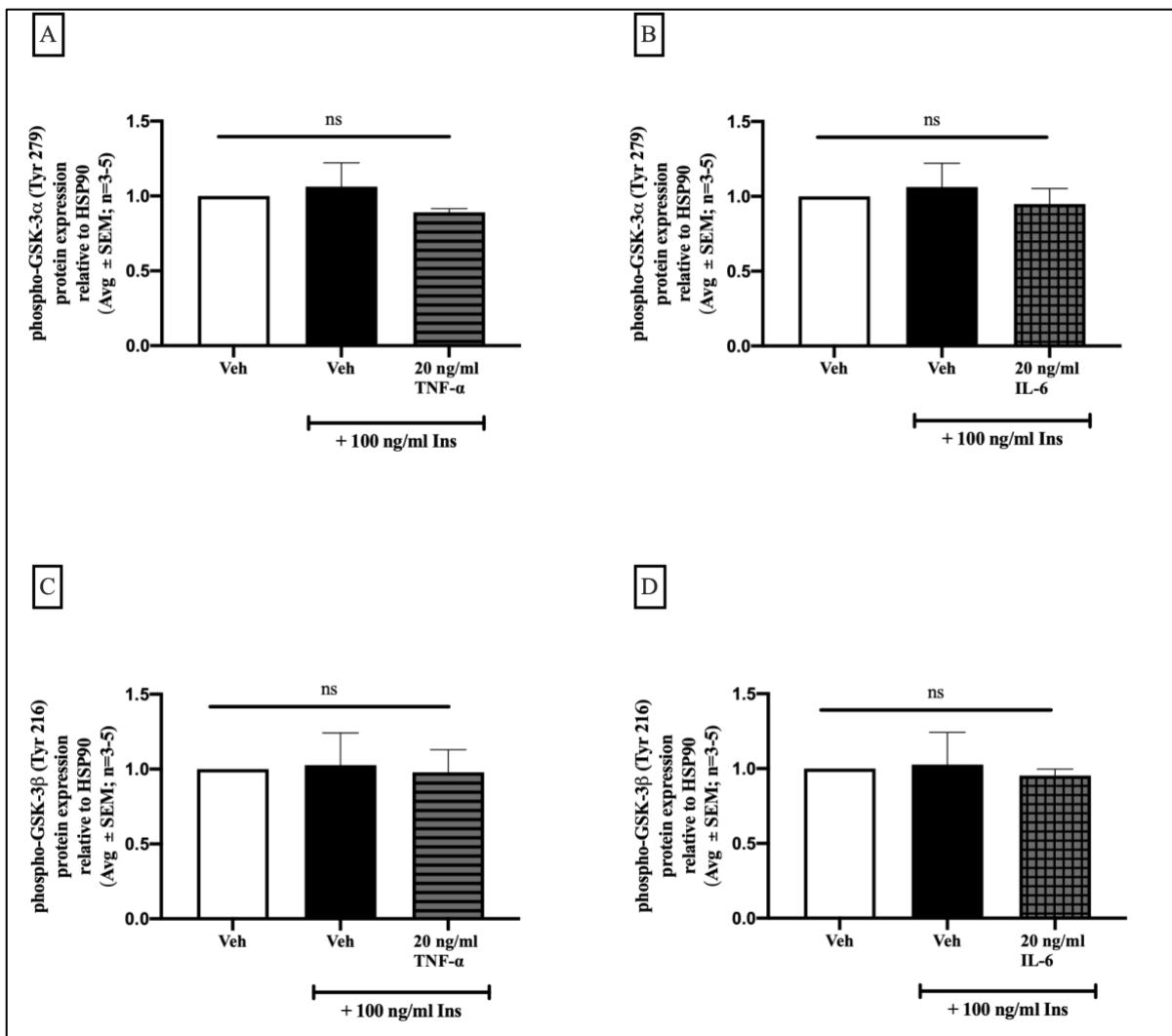


Figure 3.19. Neither insulin nor the cytokines affect the tyrosine phosphorylation of GSK-3 in the HepG2 cell line. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Tyrosine phosphorylation of GSK-3 α (Tyr279) (A, B) and GSK-3 β (Tyr216) (C, D) was measured and quantified using HSP90 as the loading control. Images are separated by cytokine type, i.e TNF- α (A, C) and IL-6 (B, D). Data shown represents between three and five independent experiments. Statistical analysis was performed using an unpaired student's t-test; ns represents no significance.

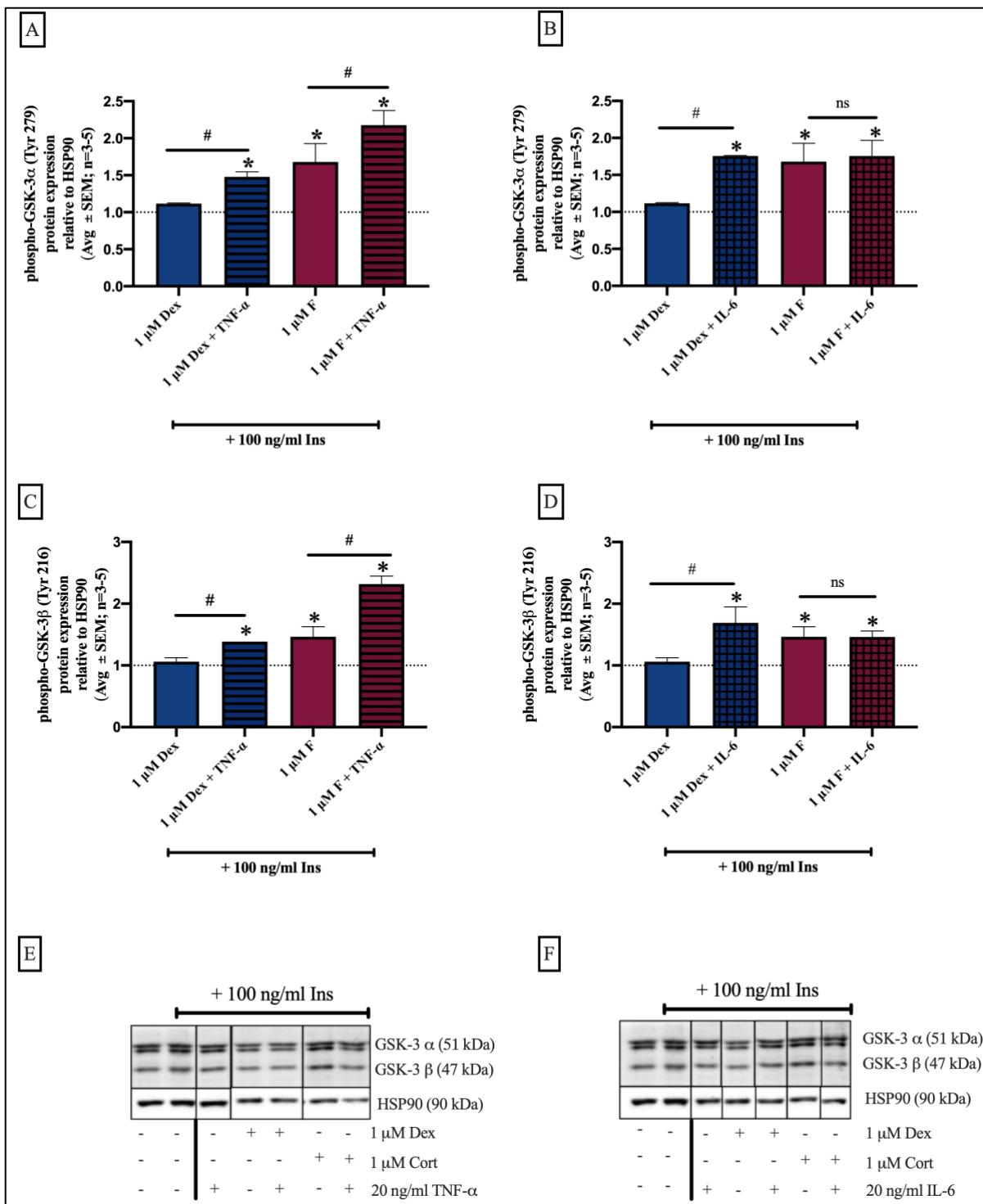


Figure 3.20. TNF- α displays co-operative behaviour in the presence of both dexamethasone (Dex) and cortisol (Cort), increasing phosphorylation of GSK- α and GSK-3 β in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 1 μ M Dex or corticosterone (Cort), in the absence or presence of 20 ng/ml of either TNF- α or IL-6. This was done for 24 hours. Cells were then treated with 100 ng/ml insulin (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. Tyrosine phosphorylation of GSK-3 α (Tyr279) (A, B) and GSK-3 β (Tyr216) (C, D) was measured and quantified using HSP90 as the loading control. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (A, C, E) and IL-6 (B, D, F) Data shown represents between three and five independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p<0.05$) representing comparison to the insulin control only whilst # ($p<0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p>0.05$) represents no significance.

3.6 Effects of the test compounds on the mRNA expression of G6Pase

G6Pase plays an important role in both gluconeogenesis and glycogenolysis as both metabolic pathways produce its substrate, glucose-6-phosphate (283). This key metabolic enzyme thus affects blood glucose homeostasis through the hydrolysis of glucose-6-phosphate producing glucose, which can enter into the blood (110). For this reason, the effect of the synthetic glucocorticoid dex in the absence and presence of the pro-inflammatory cytokines on G6Pase mRNA expression was investigated in hepatoma cell lines stimulated with insulin for 30 minutes.

In the murine BWTG3 cell line, stimulation with 100 ng/ml insulin for 30 minutes in the absence of pro-inflammatory cytokine treatment did not affect G6Pase mRNA expression. Similarly, in the presence of 20 ng/ml TNF- α , no change in the mRNA expression of G6Pase was observed. In contrast, insulin in the presence of 20 ng/ml IL-6 significantly ($p<0.05$) decreased G6Pase mRNA expression (Fig. 3.21.A).

Dex, only at the lowest concentration tested (10 nM) and in the absence of pro-inflammatory cytokine treatment, significantly ($p<0.05$) increased G6Pase mRNA levels. No significant ($p>0.05$) change in the mRNA expression of G6Pase was observed in response to higher dex (100 nM and 1 μ M) treatment (Fig. 3.21.B & C).

TNF- α (20 ng/ml) significantly ($p<0.05$) inhibited the induction of G6Pase mRNA in response to 10 nM dex by 3-fold (Fig. 3.21.B). However, at the higher concentrations of dex, the presence of 20 ng/ml TNF- α did not significantly ($p>0.05$) change the mRNA expression of G6Pase compared to dex only treatment (Fig. 3.21.B).

Whilst 20 ng/ml IL-6 had no significant ($p>0.05$) effect on dex treatment at all three concentrations tested in BWTG3 cells, a slight decrease in G6Pase mRNA levels was observed with 10 nM dex treatment to levels not statistically different from basal levels (Fig. 3.21.C).

Unlike with the BWTG3 cells, treatment of the HepG2 cells with 100 ng/ml insulin for 30 minutes resulted in a significant ($p<0.05$) decrease in G6Pase mRNA levels (Fig. 3.22A). The insulin-mediated reduction in G6Pase mRNA expression was opposed in the presence of 20 ng/ml of TNF- α or IL-6 (Fig. 3.22.A)

Dex, at all three concentrations tested, significantly ($p<0.05$) increased G6Pase mRNA expression in the HepG2 cell line thereby opposing the insulin-induced repression of G6Pase mRNA levels (Fig. 3.22. B & C). The addition of 20 ng/ml TNF- α was only able to antagonise

the effect of 10 nM dex, whilst at higher dex concentrations (100 nM and 1 μ M) it was unable to prevent the dex-induced increase in G6Pase mRNA levels, although levels induced by co-treatment with TNF- α at high dex concentrations was also not significantly ($p>0.05$) different to that of insulin only treatment (Fig. 3.22.B). In contrast to TNF- α , IL-6 had no significant ($p>0.05$) effect on dex-induced increase G6Pase mRNA levels at all concentrations tested. However, like with TNF- α co-treatment, G6Pase mRNA levels in response to 100nM- and 1 μ M dex in the presence of IL-6 was not statistically different to the insulin only treatment (Fig. 3.22C).

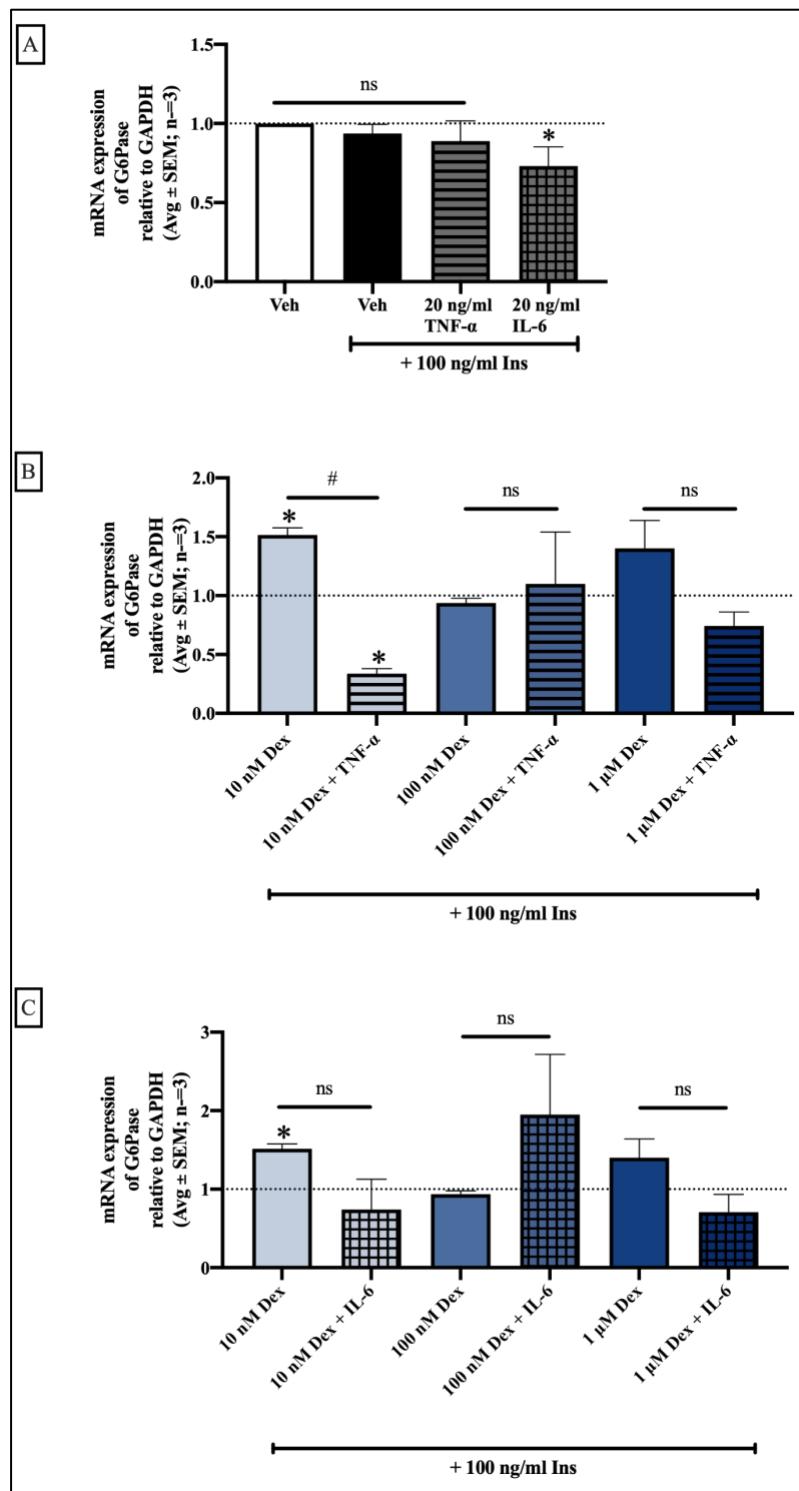


Figure 3.21. TNF- α antagonised dex-induced increase of G6Pase mRNA expression only at low concentrations of dex in BWTG3 cells. BWTG3 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dex, or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. G6Pase mRNA expression was measured and normalised using GAPDH as the housekeeping gene. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (**B**) and IL-6 (**C**). Data shown represents three independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

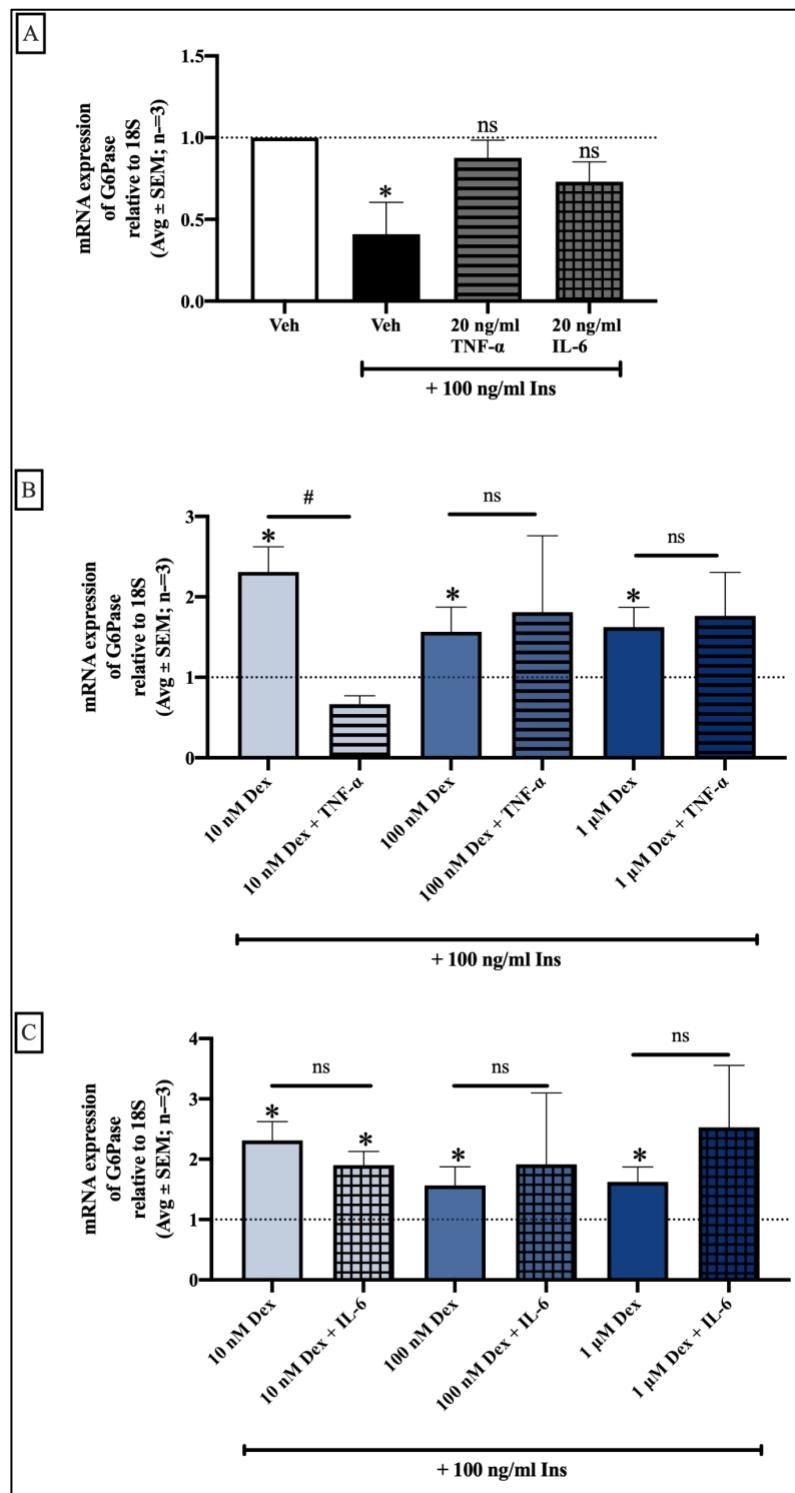


Figure 3.22. TNF- α antagonised dex-induced increase of G6Pase mRNA expression only at low concentrations of dex in HepG2 cells. HepG2 cells were serum starved for 24 hours. Thereafter the cells were treated with 10 nM, 100 nM or 1 μ M dex, or 20 ng/ml of either TNF- α or IL-6. For co-treatments, cells 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 (Ins) for 30 minutes before lysis to stimulate the insulin signalling pathway. G6Pase mRNA expression was measured and normalised using 18S as the housekeeping gene. The response of insulin was set to 1 (represented by the dotted line) and all treatments were normalised to this insulin control. Images are separated by cytokine type, i.e TNF- α (B) and IL-6 (C) Data shown represents three independent experiments. Statistical analysis was performed using an unpaired student's t-test with * ($p < 0.05$) representing comparison to the insulin control only whilst # ($p < 0.05$) represents comparing the GC treatment in the absence and presence of the pro-inflammatory cytokine; ns ($p > 0.05$) represents no significance.

CHAPTER FOUR: DISCUSSION & CONCLUSIONS

4.1 Introduction

In recent years, diabetes has become a huge threat to global health and a burden on healthcare systems (284).

The liver is an important organ, which releases glucose into circulation in the absence of insulin, however, in the presence of insulin, *de novo* glucose synthesis is halted and available glucose stored in the form of glycogen (107). Decreased inhibition of hepatic glucose production due to insulin resistance is a key contributing factor to the development and progression of T2DM, which accounts for over 90% of all diabetes cases worldwide (285–287). Hepatic insulin resistance can be described as the decreased sensitivity of the liver to insulin, leading to hepatic glucose production and consequently hyperglycaemia, both key features of diabetes mellitus (10, 11, 288). Many factors have been linked to the aetiology of insulin resistance. These include a chronic stressed state (represented by an increase in either endogenous or exogenous GCs) (9, 16, 17) as well as a chronic inflammatory state (9, 18, 21). The fact that these two events serve opposing primary functions in the body, yet still result in a similar fate, lead us to question whether the biological mediators responsible for these processes (GCs and pro-inflammatory cytokines) are able to co-regulate the insulin signalling pathway.

As mentioned, in Chapter 1 (section 1.1), the insulin signalling pathway plays an important role in the development of insulin resistance. It has been established that changes in the expression and phosphorylation state of any of the key nodes can act as contributing factors (6, 10–15). For the purpose of this study, the key node under investigation was the signalling protein AKT and its downstream target GSK-3, which is involved in glycogen synthesis. Furthermore, the mRNA expression of a key gluconeogenic enzyme, G6Pase was also investigated. In order to investigate co-regulation, the effects of the test compounds on their own in the presence of insulin had to be measured to establish baselines to which co-treatment of a GC with a pro-inflammatory cytokine could be compared.

4.2 Effects of the test compounds on the protein expression and activation of AKT

The PI3K/AKT pathway plays a critical role in normal metabolism. AKT serves as the central protein in the pathway, controlling the regulation of a number of metabolic processes including

hepatic glucose production, glycogenolysis, glycogen synthesis as well as fatty acid synthesis (141, 289). The expression and regulation of this serine/threonine kinase is critical to the functioning of the metabolic branch of the insulin signalling pathway (141). Loss of or deactivating of this protein leads to reduced insulin signalling capacity and ultimately results in obesity, insulin resistance, and finally T2DM (141, 290). For this reason, the current study looked at the protein expression as well as regulatory phosphorylation of AKT in response to co-treatment with GCs and a pro-inflammatory cytokine.

4.2.1 AKT protein expression

The total AKT protein levels are directly correlated to the amplification of the downstream signalling of the insulin signalling pathway. Influencing the levels of AKT will influence the extent of insulin signal transduction and as a result the progression to an insulin resistant state. In the current study, the pro-inflammatory cytokines did not influence the inability of insulin to affect total AKT protein levels in both the murine and human hepatoma cell lines (Figs 3.1A & B and 3.2 A & B). Similarly, a study conducted by Osawa, *et al.*, showed by western blotting that treatment of hepatocytes with 20 ng/ml TNF- α , did not affect AKT protein levels, although this was in the absence of insulin (291). In contrast, another study observed, also using western blotting, that HepG2 cells treated with 20 ng/ml IL-6 in the presence of either 0.5 nM or 100 nM insulin exhibited increased total AKT protein expression, which appeared to be time dependent although the western blots were not quantified and only one representative figure was shown (34). Similarly, Kim JH *et al.*, also using HepG2 cells and western blotting, which was not quantified, demonstrated that insulin slightly decreased total AKT expression, which was restored by IL-6 to that of basal AKT protein levels (292). These studies used a similar IL-6 concentration to that of the current study (20ng/ml) however, the maximum treatment time with IL-6 reported, was much shorter (between 1 and 8 hours) compared to the 24 hrs treatment of the current study. This might suggest that the effect of IL-6 on AKT might be time sensitive and that recovery of affected AKT expression is quickly restored to basal levels, although this hypothesis requires further investigation.

Unlike for the pro-inflammatory cytokines, the effect on AKT expression by the GCs appeared to be cell line dependent, in addition to the type of GC used i.e., natural vs synthetic. Firstly, treatment with the synthetic GC dex had no effect on AKT protein levels in the murine cell line (Figs 3.1 C&D), however, resulted in increased AKT expression in the human hepatoma cell line (Figs 3.3 C&D). Conversely, the treatment with the respective natural GCs (corticosterone for the murine cell line and cortisol for the human cell line) resulted in decreased AKT protein

levels in the BWTG3 cell line (Figs 3.2 C&D) but had no effect in the HepG2 cell line (Figs 3.4 C&D). The results suggest that the BWTG3 cell line is likely more sensitive to its endogenous GC, corticosterone whereas the HepG2 cell line is likely more sensitive to treatment with the exogenous GC, dex. This cell line specific effects could be attributed to differential steroid receptor expression levels as the endogenous GCs are known to also signal *via* steroid receptors other than the GR (293).

The literature on the effects of GCs on the protein expression of AKT is conflicting with no literature available, to the best of our knowledge, on the effects of GCs on AKT protein levels in the liver. However, AKT protein expression in muscle cells have been investigated, with two such *in vivo* studies observing either no change in muscle tissue after rats were treated with 1 mg/kg dex for 12 days (294) or decreased AKT protein expression following treatment with 1 mg/kg dex for 11 days (17). In contrast, a study performed by Kim & Park, *et al.*, reported an increase in AKT expression after treatment with dex at the same GC concentrations used in the current study (219). Their study was, however, performed in C2C12 mouse muscle cells, treated with dex for a period of 48 hours. The study also lacks insulin-stimulation of the insulin signalling pathway, which could possibly have influenced the outcome in addition to length of treatment (219). Corticosterone treated of rats in contrast, have been demonstrated to decrease AKT protein levels in the hippocampus (135), which aligns with the general trend observed in the BWTG3 cells exposed to corticosterone in the current study.

Co-treatment with the GCs and pro-inflammatory cytokines suggested that co-regulation does occur at this node, albeit pro-inflammatory cytokine specific, GC specific as well as cell line specific. There appears to be a tendency whereby co-treatment increases AKT protein expression in the BWTG3 cells (Figs 3.1 D and 3.2 C), whilst decreasing AKT protein levels in HepG2 cells (Fig 3.3 D).

In the BWTG3 cells, the pro-inflammatory cytokines could co-regulate AKT expression in the presence of both the endogenous and exogenous GCs (Figs 3.1 D and 3.2 C), however, only TNF- α had the ability to do so in the presence of corticosterone (100 nM and 1 μ M) and IL-6 only in the presence of the highest concentration of dex (1 μ M). Whilst neither 1 μ M dex nor IL-6 alone had any significant ($p>0.05$) effect on AKT protein expression (Figs 3.1 B, C & D), co-treatment with these two signalling molecules significantly ($p<0.05$) increased total AKT protein expression, suggestive of cooperativity (Fig 3.1 D). TNF- α , on the other hand was able

to antagonise the effects of corticosterone in the BWTG3 cell line (Fig 3.2 C) displaying its typical opposing character towards GCs.

In the HepG2 cells, IL-6 is the only cytokine that could co-regulate AKT protein levels and only in the presence of the exogenous GC, dex (Fig 3.3 D). No co-regulation occurred in response to treatment with the endogenous GC, cortisol in the presence of either of the cytokines in the human liver cell line (Fig 3.4 C&D). IL-6 is therefore able to oppose the dex-induced increase in AKT expression, allowing for AKT protein levels to return to basal, once again displaying restorative action similar to that described above in the study performed by Kim JH, *et al* (292).

Thus, the current study has shown that AKT is a node of the insulin signalling pathway, which is susceptible to co-regulation albeit GC specific, cytokine specific and cell-line specific. This is important to consider as changes in AKT protein levels will influence further downstream signalling which will be considered in the next sections.

4.2.2 Phosphorylation of AKT at threonine 308

AKT is phosphorylated at its threonine 308 residue by PDK-1 after activation of the insulin signalling pathway. This residue is also vulnerable to dephosphorylation by protein phosphatase 2A (Fig 1.1) (295). Phosphorylation at this site is essential for the activation of AKT and subsequent actions of the insulin (90, 95, 279). A study by Wei, *et al.*, concluded that a mutation of AKT at this site to alanine could lead to decreased AKT protein levels, increase degradation of the protein as well as prevent the release of the enzyme from the plasma membrane following exposure to insulin (296) the consequences being dysregulation of the PI3K/AKT pathway of insulin signalling (44, 296).

An increase in phosphorylation of AKT at threonine 308 is expected after treatment with insulin (297, 298) and was indeed observed in both cell lines, with similar fold induction at this phosphorylation site observed (Figs 3.5 – 3.8 A&B). Neither of the cytokines had the ability to affect the insulin-induced phosphorylation in either of the cell lines (Figs 3.5 – 3.8 A&B). To the best of our knowledge, phosphorylation of AKT at threonine 308 after treatment with IL-6 has not been investigated and therefore the results of this study cannot be compared to literature. With regards to TNF- α , Sandra, *et al.*, found that treatment of a human squamous cell carcinoma cell line (SAS cells) with 10 ng/ml of the cytokine increased insulin-induced (100 ng/ml insulin for 5 min) phosphorylation of AKT at threonine 308 (298). This effect was, however, seen at shorter treatment times compared to the current study (1 hour or less versus

24 hours) and a restoration toward basal phosphorylation was observed after longer treatment durations (between 10 and 30 hours). This data suggests TNF- α is perhaps more effective to increase AKT phosphorylation at threonine 308 at shorter treatment times and these should be investigated in the hepatocyte cell models. However, the decrease to basal levels with longer exposure times to the pro-inflammatory cytokines observed by Sandra, *et al.*, could possibly suggest that even longer exposure time might negatively affect insulin-induced threonine 308 phosphorylation (298).

Following treatment with dex, no effect was observed in the BWTG3 cell line (Figs 3.5 C&D). In contrast, treatment with dex increased the insulin-induced phosphorylation of threonine 308 in the HepG2 cell line (Fig 3.7 C&D). Again, highlighting cell line specific effects and that the HepG2 cell line is more sensitive to dex than the BWTG3 cell line. The increase in AKT threonine 308 phosphorylation in the human hepatoma cell line is in contrast to a study performed by Andrade, *et al.*, where a decrease in threonine 308 phosphorylation was observed after treatment with dex (218). However, this study was performed in rat mast cells, treated with dex for 18 hours, in the absence of insulin stimulation and in the presence of IgE directed antigen (218). However, the increase in insulin-induced threonine 308 phosphorylation seen in dex-treated HepG2 cells correlates to the increase in total AKT expression and it is therefore likely that the increased phosphorylation is due to the increased AKT protein expression (Figs 3.3 C&D). The murine endogenous GC, corticosterone, like dex, had no effect on AKT phosphorylation at threonine 308 in the BWTG3 cell line, except at 1 μ M where it significantly ($p<0.05$) decreased insulin-induced phosphorylation. This decrease could be attributed to the decrease in total AKT expression (Fig 3.2 B&C). The human endogenous GC, cortisol displayed no ability to significantly ($p>0.05$) affect insulin-induced phosphorylation of AKT at threonine 308 in HepG2 cells (Figs 3.8 C&D). Literature pertaining to the effect of these natural GCs on AKT threonine 308 phosphorylation was, however, not found and a comparison cannot be made.

In regard to possible co-regulation, co-treatment with dex in the presence of either TNF- α or IL-6, no significant ($p>0.05$) co-regulation of AKT phosphorylation at threonine 308 was observed in either of the two cell lines (Figs 3.5 and 3.7 C&D). Co-treatment with the endogenous GC, corticosterone and the pro-inflammatory cytokines exhibits no significant ($p>0.05$) co-regulation in the BWTG3 cells except at the highest concentration of corticosterone used (Figs 3.6 C&D), where both pro-inflammatory cytokines antagonised corticosterone's ability to decrease insulin-induced threonine 308 AKT phosphorylation. IL-6

was further able to augment insulin-stimulated AKT phosphorylation at this residue (Fig. 3.6 D). In the HepG2 cells, only co-treatment with 100 nM cortisol and of IL-6 resulted in a significant ($p<0.05$) decrease in the insulin-induced phosphorylation of threonine 308 (Fig 3.8 D). Like with total AKT levels no literature is available (to the best of our knowledge) describing AKT phosphorylation at threonine 308 in response to co-treatment with GCs and pro-inflammatory cytokines. The results again indicate GC-, cell line, and pro-inflammatory cytokine specific effects.

4.2.3 Phosphorylation of AKT at serine 473

Following AKT phosphorylation at threonine 308, phosphorylation at serine 473 is also observed. This residue is phosphorylated by mTORC2 and phosphorylation at this site is required for full activation of the kinase activity of AKT (44, 46, 47). Lack of serine 473 phosphorylation still renders AKT active, however, it displays reduced kinase activity. This is due to the fact that serine 473 phosphorylation is required for the stabilization of threonine 308 phosphorylation and subsequently the activation state of AKT (299, 300). Dephosphorylation of the serine 473 residue of AKT occurs *via* PP2A as well as PHLPP (Fig 1.2) (84, 85). Mutation of this amino acid to alanine lead to dysregulation of the AKT pathway similar to what was found for the threonine 308 residue (44, 296).

Like with the threonine 308 site, an increase in phosphorylation of AKT at the serine 473 site is expected after treatment with insulin due to activation of the pathway (294, 297, 298). In the current study, the increase observed in the BWTG3 cell line (Figs 3.9 and 3.10 A&B) was more pronounced than in the HepG2 cell line (Figs 3.11 and 3.12 A&B), with the BWTG3 cell line reaching a maximal induction of 31-fold (Fig 3.10 A&B) compared to the 17-fold maximal induction observed in the HepG2 cells (Fig 3.12 A&B). This difference in insulin-induced phosphorylation of AKT at serine 473 in the two cell lines could possibly be explained by disparate PP2A or PHLPP levels or activity. Neither of the pro-inflammatory cytokines had the ability to affect the insulin-induced phosphorylation at serine 473 in either of the cell lines (Figs 3.9 – 3.12 A&B). Contrary to the results seen in the current study, the study by Sandra, *et al.*, showed that treatment with 10 ng/ml TNF- α could increase insulin-induced phosphorylation of AKT at the serine 473 site in SAS cells with treatment times ranging from 1 minute up to 30 hours (298). However, similar to the current study, no change in AKT phosphorylation at serine 473 in human vascular endothelial cells treated with IL-6 was observed in a study done by Zegeye, *et al* (301).

Treatment with all concentrations of the exogenous GC (dex) did not influence the ability of insulin to induce phosphorylation of ATK at serine 473 in the BWTG3 cell line (Figs 3.9 C&D) whereas augmentation of insulin-stimulated phosphorylation of serine 473 in the HepG2 cells was demonstrated (Figs 3.11 C&D). Previous reports have shown that dex increases phosphorylation of AKT at serine 473 in either mast cells (218) or the C2C12 mouse myoblasts cell line (219). The difference in cell type, treatment time as well as the lack of insulin stimulation may account for the differences seen between the current study and the above-mentioned studies. In contrast to dex, a general increase in insulin-induced phosphorylation of AKT at the serine 473 was observed after treatment with the endogenous GCs in both cell lines, however, this increase was only significant ($p<0.05$) at 1 μ M (Fig 3.10 and 3.12 C&D). A similar result was observed after treatment of C2C12 muscle cells with cortisone, the inactive form of the endogenous human GC (219).

Concerning AKT phosphorylation at serine 473 in response to co-treatment with the GCs and pro-inflammatory cytokines some co-operative effects were observed. In the BWTG3 cell line, generally both the synthetic and endogenous GC co-treated with the pro-inflammatory cytokines portrayed co-operative activity by potentiating the insulin-induced AKT phosphorylation at serine 473, except for 1 μ M corticosterone in the presence of IL-6 and 1 μ M dex in the presence of TNF- α , where antagonism of the GC response was observed in response to the pro-inflammatory cytokine (Fig. 3.9 C&D and Fig.3.10 C&D). This increase suggests a co-operative effect between the GC and the pro-inflammatory cytokine as on their own, neither generally induced a response (Fig 3.9 A–D). The endogenous hormone corticosterone exhibited cooperativity with TNF- α , however, only at the two highest concentrations tested probably due to its lower potency *via* the GR (Fig.3.10 C). Taken together this would suggest that cooperativity between the GCs and pro-inflammatory cytokines is concentration dependent and in terms of corticosterone also cytokine specific.

Curiously co-treatment with dex and both pro-inflammatory cytokines in the HepG2 cells only portrayed cooperativity at the highest (1 μ M) dex concentration used, whereas antagonism by the cytokines were observed at the lower concentrations dex (Fig. 3.11 C&D), again, highlighting the dependence of cooperativity on the concentration of GC present in addition to the cell line used when considering insulin stimulated AKT phosphorylation of serine 473 by GCs and cytokines.

To summarise, a general increase in insulin-induced phosphorylation of AKT at either site following co-treatment with TNF- α was observed in the BWTG3 cells, regardless of the GC involved. In the HepG2 cells, TNF- α has the ability to increase or decrease insulin-induced phosphorylation of AKT at serine 473, regardless of the GC present. Furthermore, at serine 473 in the HepG2 cells, the cytokines act in a similar manner to one another when co-treated with dex. Co-treatment with IL-6 generally leads to a decrease in the insulin-induced phosphorylation of AKT at both the threonine 308 and serine 473 site. However, IL-6 displays the ability to increase phosphorylation in both cell lines and in the presence of either of the GCs and dependent on the concentration GC used. Together the results suggest that AKT may be considered a node within the insulin signalling pathway that is co-regulated by stress and inflammatory mediators. The test compounds appear to have the greatest potential of co-regulating the phosphorylation at serine 473 rather than threonine 308. This difference suggests that they perhaps have the ability to affect mTORC2 responsible for the phosphorylation of AKT at serine 473. Downstream of AKT is GSK-3, which is inhibited *via* phosphorylation by activated AKT and might be affected by the co-regulation observed for AKT by the GCs and pro-inflammatory cytokines.

4.3 Effects of the test compounds on the protein expression and tyrosine phosphorylation of GSK-3

The serine/threonine kinase GSK-3 has multiple substrates including GS, the enzyme responsible for the synthesis of glycogen, which it phosphorylates and subsequently inactivates (50, 51). Active GSK-3 is therefore important for the inhibition of the enzyme, leading to decreased glycogen synthesis. Additionally, GSK-3 is also responsible for the inactivation of IRS-1 thereby leading to disruption in insulin signal transduction (302) GSK-3 is considered to be auto-phosphorylated at specific tyrosine residues, which is responsible for its kinase activity (49).

4.3.1 Protein expression of GSK-3

Dysregulation of GSK-3 has been implicated in the disease progression of T2DM (50, 122–125). A lack of GSK-3 β , in the presence of GSK-3 α in mouse models results in the death of these mice in the early stages of embryo development, whereas the opposite (GSK-3 β present with a lack of GSK-3 α) improved insulin sensitivity and glucose tolerance (303, 304). For this

reason, it is safe to assume that GSK-3 β is the subtype of the enzyme most important with regards to insulin signalling.

In the current study, stimulation with insulin had no effect on the GSK-3 α or GSK-3 β protein levels in both the murine and human hepatoma cell line (Figs 3.13 A-D and 3.15 A-D). Similarly, the presence of either of the pro-inflammatory cytokines did not influence GSK-3 protein expression for either of the subtypes (Figs 3.13 A-D and 3.15 A-D). Wang and colleagues demonstrated (albeit only with one unquantified blot) that treatment with 20 ng/ml TNF- α did not affect the GSK-3 protein expression (305). However, this was in the PC3 prostate cancer cell line as the effects of TNF- α and IL-6 on the protein expression of GSK-3 has not been investigated in hepatocytes, to the best of our knowledge.

Neither of the GCs affected the protein expression of GSK- α in the murine and human hepatoma cell lines (Figs 3.14 A&B and Figs 3.16 A&B). In contrast, treatment with both the endogenous and exogenous GCs increased protein expression of the GSK-3 β subtype in the BWTG3 cell line (Figs 3.14 C&D), whilst only the natural ligand, cortisol, increased the protein expression of the beta subtype of the enzyme in the HepG2 cells (Figs 3.16 C&D). Similar to our results, GSK-3 α protein levels were unaffected in muscles of dex-treated rats, although the authors of this study also observed no effect on GSK-3 β protein expression, which was not demonstrated in the current study (294). These results suggest that the GCs might influence insulin signalling by increasing GSK-3 β protein levels.

Similar to the individual treatments, co-treatment with the GCs and pro-inflammatory cytokines had no effect on the protein levels of GSK-3 α in both the murine and human hepatoma cell lines, although a slight decrease in GSK-3 α levels were observed when dex was co-treated with IL-6 (Figs 3.14 A&B and Figs 3.16 A&B). Thus, only co-treatment with dex and IL-6 can be considered to exhibit co-regulation of GSK-3 α protein levels, even though only a small but significant ($p<0.05$) decrease was shown (Fig.3.14 B). In contrast, increased GSK-3 β protein levels in response to the endogenous GCs in both murine and human hepatoma cell lines were generally antagonised by IL-6 (Fig. 3.14 D and Fig. 3.16 D), whilst co-treatment with TNF- α had no effect (Fig.3.14 C and Fig. 3.16 D). Antagonism of corticosterone-induced GSK-3 β protein expression by IL-6 in the murine hepatoma cell line especially was quite pronounced as GSK-3 β protein levels were decreased by approximately 40% below basal levels (Fig. 3.14 D). Although no significance could be established, dex-mediated increase of GSK-3 β protein levels was decreased to basal levels in BWTG3 cells (Fig. 3.14). Taken

together TNF- α , which exhibited no co-regulation, will have no influence on the GCs to influence insulin signalling *via* GSK-3 β , whereas IL-6 could potentially obstruct this, although in a cell line specific manner.

4.3.2 Tyrosine phosphorylation of GSK-3

GSK-3 is highly phosphorylated at key tyrosine residues in its active state, however, the mechanism of regulation of this phosphorylation event is not yet well understood (306). Autophosphorylation is one proposed mechanism (307). Phosphorylation at these sites is crucial for its kinase activity as mutations at these residues to alanine impairs the function of the protein (48, 121). Active GSK-3 causes the phosphorylation of GS, decreasing glycogen synthesis, as well as inactivating IRS-1 through serine phosphorylation (302, 308). T2DM has been linked to a decrease in GS activity and glycogen synthesis, as well as the inability of IRS-1 to take part in the insulin signalling cascade, which is attributed to dysfunctional GSK3 signalling (50, 122–124).

Just as with the protein expression of GSK-3, no change was observed in the tyrosine phosphorylation after treatment with insulin in the absence or presence of either of the pro-inflammatory cytokines in both the murine and human hepatoma cell lines (Figs 3.17 A-D and 3.19 A-D). Similarly, dex had no effect on the phosphorylation of GSK-3 α or GSK-3 β in both cell lines used (Figs 3.18 A-D and 3.20 A-D), whereas the endogenous GC exhibited cell line specific effects. In the BWTG3 cell line, the endogenous GC, corticosterone, decreased tyrosine phosphorylation of both GSK-3 subtypes (Figs 3.18 A-D) whilst treatment with the endogenous GC, cortisol, resulted in increased tyrosine phosphorylation of both GSK-3 subtypes in the HepG2 cells (Figs 3.20 A-D). To the best of our knowledge, no studies have investigated the effects of GCs on the tyrosine phosphorylation of GSK-3 and therefore, we cannot make a comparison to the literature. Increased GSK-3 tyrosine phosphorylation of both subtypes is indicative of a more catalytically active enzyme and thus the endogenous GC in the human hepatoma cell line could potentially greatly influence GS activity and negatively influencing insulin signalling *via* IRS-1. This however would not be observed in the BWTG3 cells due to the decrease in tyrosine phosphorylation observed, although further investigation is required to test this hypothesis.

When looking at the co-treatment of the GCs with the pro-inflammatory cytokines, TNF- α in the presence of the GCs generally either increased tyrosine phosphorylation of both GSK-3 subtypes or had no effect compared to GC treatment only (Fig. 3.18 A&C and Fig. 3.20 A&C).

In the murine hepatoma cell lines, although no significant ($p>0.05$) co-regulation could be established, GC co-treatment with TNF- α restored tyrosine phosphorylated levels of both GSK-3 subtypes to basal levels and in the case of GSK-3 β , co-treatment with dex significantly ($p<0.05$) increased it above basal (Fig. 3.18 A&C). In contrast, in the HepG2 cells, TNF- α with both endogenous and synthetic GC exhibited cooperative regulation, increasing tyrosine phosphorylation of both GSK-3 subtypes (Fig. 3.20 A&C). Unlike TNF- α , co-treatment with IL-6 elicited differential effects depending on the GC as well as the cell line used (Fig. 3.18 B&D and Fig.3.20 B&D). Corticosterone-induced inhibition of tyrosine phosphorylation of both GSK-3 subtypes was antagonised by IL-6 back to basal levels in BWTG3 cells (Fig. 3.18 B&D), whilst dex co-treatment with IL-6 had no effect on basal tyrosine phosphorylation of GSK-3 α , this co-treatment did decrease basal GSK-3 β tyrosine phosphorylation, suggesting that exposure to dex and IL-6 could possibly negatively affect GSK-3 β signalling and subsequent GS and IRS-2 inactivation.

In contrast to the co-treatments in the BWTG3 cells, GCs co-treated with the pro-inflammatory cytokines generally exhibited co-operative effects in the HepG2 cells, except for IL-6 co-treated with cortisol where no cooperativity was observed (Fig.3.20). Nonetheless, co-treatment conditions with either cytokine significantly ($p<0.05$) increased tyrosine phosphorylation of both GSK-3 subtypes indicative of a more catalytically active GSK-3 in the HepG2 cell line. Increased GSK-3 β tyrosine phosphorylation also holds great importance as it is said to have more potential substrates than any other kinase (281, 309). The mechanism responsible for this increase in phosphorylation of GSK-3 subtypes at their respective tyrosine residues remains to be elucidated and could potentially involve increased intracellular calcium levels, which have been reported to induce tyrosine phosphorylation of GSK-3 β (310). Additionally, cAMP levels could be increased by this co-treatment, which have also been shown to activate GSK-3 via a cAMP-activated protein tyrosine kinase (311). These could serve as future investigations into understanding the molecular mechanism involved in the co-operative regulation of GSK-3 by the GCs and pro-inflammatory cytokines.

4.4 Effects of the test compounds on the mRNA expression of G6Pase

One of the main functions of insulin in the liver is the inhibition of glucose production either from gluconeogenesis or glycogenolysis. The final reaction within these two pathways is catalysed by G6Pase, which hydrolyses glucose-6-phosphate releasing glucose, thereby increasing blood glucose levels (112, 113). The protein expression of G6Pase has been reported

to significantly increase during starvation, as well as T2DM (312). G6Pase activity as well as levels are negatively affected by insulin (106, 283) and thus could be considered another node (or target) within the insulin signalling pathway. Uncontrolled increased expression of the gene encoding this protein could lead to elevated hepatic glucose production and hyperglycaemia, which ultimately leads to T2DM.

Whilst insulin has been reported to decrease G6Pase levels (313), in this study this was only observed in the HepG2 cells as insulin stimulation for 30 minutes was unable to decrease G6Pase mRNA expression in the BWTG3 cells (Fig 3.21 A and Fig. 3.22). This would suggest that downstream signalling of insulin is much less responsive in the murine hepatoma cell line than in the HepG2 cells, at least when considering the pathway involved in G6Pase regulation i.e., FOXO1 regulation. Future studies could potentially consider exposing the BWTG3 cells to insulin for longer, which might be required to successfully decrease G6Pase mRNA.

Regarding the effect of the pro-inflammatory cytokines on G6Pase mRNA expression, TNF- α had no effect on G6Pase mRNA levels in either of the cell lines, although it did restore the insulin-induced decrease of G6Pase mRNA levels to basal expression in the HepG2 cells, although significance could not be established when compared to insulin-only treatment (Figs 3.21 and 3.22 A). Similar to the current study, work done by Grempler, *et al.*, showed that treatment with 10 ng/ml TNF- α for 3 hours was able to decrease dex-induced G6Pase mRNA expression in H4IIE rat hepatoma cells, in the absence and presence of insulin (112). Furthermore, treatment of mice with 5 or 10 μ g TNF- α for 12 hours resulted in decreased G6Pase mRNA levels in the liver (314). Unlike TNF- α , IL-6 in the presence of insulin decreased G6Pase mRNA expression in the BWTG3 cell line (Fig 3.21 A). Decreased mRNA expression observed in response to IL-6 is in agreement with a study performed in mice bearing IL-6-secreting tumours (315). In contrast and similar to TNF- α , IL-6 attenuated the insulin-induced decrease in G6Pase mRNA levels to that of basal expression in the human hepatoma cell line, although not significantly ($p>0.05$) different to insulin-only treatment (Fig. 3.22 A). These results might be related to the lack of insulin-induced inhibition of G6Pase mRNA in the BWTG3 cells in comparison to the human hepatoma cell line. IL-6 might only be able to inhibit G6Pase mRNA expression when insulin is unable to affect the mRNA levels of the gluconeogenic enzyme. This could possibly be due to a different milieu of transcription factors occupying the *G6PC* promoter in the event of insulin-induced repression of the promoter compared to the lack thereof. Future studies could investigate this hypothesis by looking at the

recruitment of transcription factors associated with G6Pase regulation such as FOXO1 (97) or hepatic nuclear factor 1 (212) using chromatin immunoprecipitation assays.

GCs are known to increase G6Pase mRNA expression due to the GREs present in the *G6PC* promoter (52, 98, 109, 209, 213–215, 316). As expected, dex significantly ($p<0.05$) increased G6Pase mRNA expression at all the concentrations tested in the HepG2 cells (Fig. 3.22 B&C), whereas only at the lowest concentration used i.e., 10 nM, was able to significantly ($p<0.05$) induce G6Pase mRNA levels above basal in the murine hepatoma cell line (Fig. 3. 21 B&C). This coincides with the previously mentioned view (section 4.2.1) that the HepG2 cell line appears to be more sensitive to dex. Although it should be mentioned that in the HepG2 cells, insulin treatment resulted in a decrease in G6Pase mRNA expression, which was attenuated by dex and thus the effect of insulin or lack thereof, as is in the case of the BWTG3 cells, could possibly influence the dex-mediated effect on G6Pase mRNA expression. In addition, insulin has been reported to attenuate dex-mediated increase in G6Pase mRNA expression in Fao and H4IIE rat hepatoma cells (112), which was not observed in the current study. This, however, could be due to the concentration of insulin used as the above-mentioned studies used much higher insulin concentrations, in addition to exposing the cells to insulin for a much longer period.

In regard to the effect of co-treatment of dex with a pro-inflammatory cytokine, significant ($p<0.05$) co-regulation was only observed with TNF- α and only at the lowest concentration of dex used (10 nM) in both cell lines (Fig.3.21 B&C and Fig.3.22 B&C). TNF- α was able to antagonise the dex-mediated increase in G6Pase mRNA expression as previously reported (112) and thus would prevent hepatic glucose production. In contrast, IL-6 had no significant ($p>0.05$) effect on the dex-mediated increase in G6Pase mRNA, although generally levels returned to that of insulin-only treated levels.

To summarise, co-regulation was observed at this node of the insulin signalling pathway, albeit cytokine specific. This cytokine specific effect can be attributed to the different signalling mechanisms of these two cytokines that essentially will activate different transcription factors responsible for gene regulation (reviewed in Chapter 1, sections 1.6.1 and 1.6.2).

4.5 Conclusions and implications for future work

To conclude, the GCs and cytokines pertaining to this study were capable of co-regulating each of the different nodes of the insulin signalling investigated in this study, except GSK-3 α protein expression. However, the results are GC specific, cytokine specific as well as cell line (species)

specific. Antagonistic effects were observed, as expected, since these biological mediators primarily act in an antagonistic manner towards each other (195, 202). However, co-operative effects were also observed between the cytokines and GCs. This is not completely unexpected, as it has been shown that certain GCs and cytokines have the ability to co-regulate certain acute phase proteins (244, 259–262) although the molecular mechanism remains to be elucidated. In addition, co-regulation of the acute phase proteins is generally believed to be at the transcriptional level whereas in the current study we show that co-regulation also occurs at non-genomic events. For example, co-regulation was able to increase the insulin-induced phosphorylation effects seen at the threonine 308 and serine 473 sites of AKT, suggesting that it could potentially lead to increased insulin sensitivity as opposed to insulin resistance as expected. However, it should be noted that increased basal AKT signalling has also been implicated in insulin resistance due to an increase in lipid synthesis and oxidative stress (317). Furthermore, with increased AKT signalling, pro-longed ribosomal protein S6 kinase beta-1 (S6K) activation might occur, which is responsible for the deactivation of IRS1. This negative feedback loop is responsible for signal termination and dysregulation through increased basal AKT signalling could lead to subsequent insulin resistance.

Whether the co-regulation of these non-genomic effects are due to the transcriptional activity of the ligand-activated GR remains to be explored. Additionally, it still remains unclear whether simultaneous, rather than sequential exposure to both GCs and pro-inflammatory cytokines, which occurs *in vivo*, would result in an insulin resistant state. The current study only looked at one time point (24 hours) for all treatment condition, and longer or shorter exposure times to the test compounds might have a different effect on any insulin-mediated responses. It cannot be expected that all insulin regulated effects will become unresponsive to insulin i.e., insulin resistant, at similar exposure times. Future studies should thus also consider different treatment times. Additionally, whether co-treatment with GCs and pro-inflammatory cytokines take part in the negative feedback loop, which involves inhibitory serine residues of IR and IRS, which become phosphorylated by AKT after insulin stimulation should be investigated as excessive serine phosphorylation of these proteins would lead to disrupted insulin signalling. The effect of co-treatment with GCs and pro-inflammatory cytokines on PEPCK mRNA expression, another key gluconeogenic enzyme, as well as the protein expression and enzymatic activity, of both G6Pase and PEPCK could be explored, as mRNA expression does not necessarily equate to similar protein levels. And finally, the insulin-mediated inhibition of hepatic glucose production in response to GCs and pro-inflammatory

cytokine co-treatment should also be investigated as this is a hallmark of a hepatic insulin resistant state.

REFERENCES

1. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 37:S81–S90.
2. Pheiffer C, Pillay-van Wyk V, Joubert JD, Levitt N, Nglazi MD, Bradshaw D. 2018. The prevalence of type 2 diabetes in South Africa: a systematic review protocol. *BMJ Open* 8:e021029.
3. Mayosi BM, Flusher AJ, Laloo UG, Sitas F, Tollman SM, Bradshaw D. 2009. The burden of non-communicable diseases in South Africa. *Lancet* 374:934–947.
4. Kengne AP, Bentham J, Zhou B, Peer N, Matsha TE, Bixby H, Di Cesare M, Hajifathalian K, Lu Y, Taddei C, Bovet P, Kyobutungi C, Agyemang C, Aounallah-Skhiri H, Assah FK, Barkat A, Romdhane H Ben, Chan Q, Chaturvedi N, Damasceno A, Delisle H, Delpeuch F, Doua K, Egbagbe EE, Ati J El, Elliott P, Engle-Stone R, Erasmus RT, Fouad HM, Gareta D, Gureje O, Hendriks ME, Houti L, Ibrahim MM, Kemper HCG, Killewo J, Kowlessur S, Kruger HS, Laamiri FZ, Laid Y, Levitt NS, Lunet N, Magliano DJ, Maire B, Martin-Prevel Y, Mediene-Benchekor S, Mohamed MK, Mondo CK, Monyeki KD, Mostafa A, Nankap M, Owusu-Dabo E, Rinke de Wit TF, Saidi O, Schultsz C, Schutte AE, Senbanjo IO, Shaw JE, Smeeth L, Sobngwi E, Jérôme CS, Stronks K, Tanser F, Tchibindat F, Traissac P, Tshepo L, Tullu F, Ukoli FAM, Viswanathan B, Wade AN, Danaei G, Stevens GA, Riley LM, Ezzati M, Mbanya JCN. 2017. Trends in obesity and diabetes across Africa from 1980 to 2014: an analysis of pooled population-based studies. *Int J Epidemiol* 46:1421–1432.
5. Pickup JC, Crook MA. 1998. Is Type II diabetes mellitus a disease of the innate immune system? *Diabetologia* 41:1241–1248.
6. Wilcox G. 2005. Insulin and Insulin Resistance. *Clin Biochem Rev* 26:19–39.
7. Taylor R. 2012. Insulin Resistance and Type 2 Diabetes. *Diabetes* 61:778–779.
8. de Luca C, Olefsky JM. 2008. Inflammation and Insulin Resistance. *FEBS Lett* 582:97–105.
9. Pickup JC, Mattock MB, Chusney GD, Burt D. 1997. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* 40:1286–1292.
10. S. Zaman G. 2020. Pathogenesis of Insulin ResistanceCellular Metabolism and Related Disorders. IntechOpen.
11. Petersen KF, Shulman GI. 2006. Etiology of Insulin Resistance. *Am J Med* 119:S10–S16.
12. Hotamisligil G. 2000. Molecular mechanisms of insulin resistance and the role of the adipocyte. *Int J Obes* 24:S23–S27.
13. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J. 2002. Serine Phosphorylation of Insulin Receptor Substrate 1 by Inhibitor κB Kinase Complex. *J Biol Chem* 277:48115–48121.
14. Paz K, Hemi R, LeRoith D, Karasik A, Kanety H, Zick Y. 1997. Molecular basis for insulin resistance: elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem*

- 272:29911–29918.
15. Kahn SE, Hull RL, Utzschneider KM. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444:840–846.
 16. Qi D, Rodrigues B. 2007. Glucocorticoids produce whole body insulin resistance with changes in cardiac metabolism. *Am J Physiol Metab* 292:E654–E667.
 17. Burén J, Lai YC, Lundgren M, Eriksson JW, Jensen J. 2008. Insulin action and signalling in fat and muscle from dexamethasone-treated rats. *Arch Biochem Biophys* 474:91–101.
 18. Wellen KE, Hotamisligil GS. 2005. Inflammation, stress, and diabetes. *J Clin Invest* 115:1111–1119.
 19. Hotamisligil G, Shargill N, Spiegelman B. 1993. Adipose Expression of Tumor Necrosis Factor- α : Direct Role in Obesity-Linked Insulin Resistance. *Science* (80-) 259:87–91.
 20. Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW, Mooney RA. 2003. Suppressor of Cytokine Signaling-3 (SOCS-3), a Potential Mediator of Interleukin-6-dependent Insulin Resistance in Hepatocytes. *J Biol Chem* 278:13740–13746.
 21. Galic S, Oakhill JS, Steinberg GR. 2010. Adipose tissue as an endocrine organ. *Mol Cell Endocrinol* 316:129–139.
 22. Reynolds RM, Walker BR. 2003. Human insulin resistance: the role of glucocorticoids. *Diabetes, Obes Metab* 5:5–12.
 23. Engelking LR. 2015. Gluconeogenesis, p. 225–230. *In* Textbook of Veterinary Physiological Chemistry. Elsevier.
 24. Bauerle KT, Harris C. 2006. Glucocorticoids and Diabetes. *Mo Med* 113:378–383.
 25. Moini J. 2019. Pathophysiology of Diabetes, p. 25–43. *In* Epidemiology of Diabetes. Elsevier.
 26. Motta K, Barbosa AM, Bobinski F, Boschero AC, Rafacho A. 2015. JNK and IKK β phosphorylation is reduced by glucocorticoids in adipose tissue from insulin-resistant rats. *J Steroid Biochem Mol Biol* 145:1–12.
 27. Abdul-Ghani MA, DeFronzo RA. 2009. Plasma Glucose Concentration and Prediction of Future Risk of Type 2 Diabetes. *Diabetes Care* 32:S194–S198.
 28. Mazziotti G, Gazzaruso C, Giustina A. 2011. Diabetes in Cushing syndrome: basic and clinical aspects. *Trends Endocrinol Metab* 22:499–506.
 29. Dimitriadis G, Mitrou P, Lambadiari V, Maratou E, Raptis SA. 2011. Insulin effects in muscle and adipose tissue. *Diabetes Res Clin Pract* 93S:S52–S59.
 30. Boucher J, Kleinridders A, Kahn CR. 2014. Insulin Receptor Signaling in Normal and Insulin-Resistant States. *Cold Spring Harb Perspect Biol* 6:1–23.
 31. Tanti J-F, Ceppo F, Jager J, Berthou F. 2013. Implication of inflammatory signaling pathways in obesity-induced insulin resistance. *Front Endocrinol (Lausanne)* 3.
 32. Jager J, Grémeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF. 2007. Interleukin-1 β -induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology* 148:241–251.
 33. Kanemaki T, Kitade H, Kaibori M, Sakitani K, Hiramatsu Y, Kamiyama Y, Ito S,

- Okumura T. 1998. Interleukin 1 β and interleukin 6, but not tumor necrosis factor α , inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. *Hepatology* 27:1296–1303.
34. Senn JJ, Klover PJ, Nowak IA, Mooney RA. 2002. Interleukin-6 Induces Cellular Insulin Resistance in Hepatocytes. *Diabetes* 51:3391–3399.
35. Roytblat L, Rachinsky M, Fisher A, Greengberg L, Shapira Y, Douvdevani A, Gelman S. 2000. Raised Interleukin-6 Levels in Obese Patients. *Obes Res* 8:673–675.
36. Dandona P. 2004. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 25:4–7.
37. Jiang S, Young J, Wang K, Qian Y, Cai L. 2020. Diabetic-induced alterations in hepatic glucose and lipid metabolism: The role of type 1 and type 2 diabetes mellitus (Review). *Mol Med Rep* 22:603–611.
38. Weigert C, Hennige AM, Brodbeck K, Häring HU, Schleicher ED. 2005. Interleukin-6 acts as insulin sensitizer on glycogen synthesis in human skeletal muscle cells by phosphorylation of Ser 473 of Akt. *Am J Physiol Metab* 289:E251–E257.
39. Blumberg D, Hochwald S, Burt M, Donner D, Brennan MF. 1995. Tumor necrosis factor alpha stimulates gluconeogenesis from alanine in vivo. *J Surg Oncol* 59:220–225.
40. Goto M, Yoshioka T, Battelino T, Ravindranath T, Zeller WP. 2001. TNF α Decreases Gluconeogenesis in Hepatocytes Isolated from 10-Day-Old Rats. *Pediatr Res* 49:552–557.
41. Austin RL, Rune A, Bouzakri K, Zierath JR, Krook A. 2008. siRNA-Mediated Reduction of Inhibitor of Nuclear Factor- B Kinase Prevents Tumor Necrosis Factor- - Induced Insulin Resistance in Human Skeletal Muscle. *Diabetes* 57:2066–2073.
42. Franchimont D, Kino T, Galon J, Meduri GU, Chrousos G. 2002. Glucocorticoids and Inflammation Revisited: The State of the Art. *Neuroimmunomodulation* 10:247–260.
43. Liu Y-Z, Wang Y-X, Jiang C-L. 2017. Inflammation: The Common Pathway of Stress-Related Diseases. *Front Hum Neurosci* 11.
44. Liao Y, Hung MC. 2010. Physiological regulation of Akt activity and stability. *Am J Transl Res* 2:19–42.
45. Haeusler RA, McGraw TE, Accili D. 2018. Biochemical and cellular properties of insulin receptor signalling. *Nat Rev Mol Cell Biol* 19:31–44.
46. Świderska E, Strycharz J, Wróblewski A, Szemraj J, Drzewoski J, Śliwińska A. 2020. Role of PI3K/AKT Pathway in Insulin-Mediated Glucose UptakeBlood Glucose Levels. *IntechOpen*.
47. Summers S, Whiteman E, Birnbaum M. 2000. Insulin signaling in the adipocyte. *Int J Obes* 24:S67–S70.
48. Eldar-Finkelman H, Kaidanovich O. 2002. The role of glycogen synthase kinase-3 in insulin resistance and Type 2 diabetes. *Expert Opin Ther Targets* 6:555–561.
49. Eldar-Finkelman H. 2002. Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol Med* 8:126–132.
50. Lee J, Kim M-S. 2007. The role of GSK3 in glucose homeostasis and the development of insulin resistance. *Diabetes Res Clin Pract* 77:S49–S57.

51. Hughes K, Nikolakaki E, Plyte SEE, Totty NNF, Woodgett JRR. 1993. Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *EMBO J* 12:803–808.
52. Mitre-Aguilar IB, Cabrera-Quintero AJ, Zentella-Dehesa A. 2015. Genomic and non-genomic effects of glucocorticoids: Implications for breast cancer. *Int J Clin Exp Pathol* 8:1–10.
53. Schmoll D, Walker KS, Alessi DR, Grempler R, Burchell A, Guo S, Walther R, Unterman TG. 2000. Regulation of Glucose-6-phosphatase Gene Expression by Protein Kinase B α and the Forkhead Transcription Factor FKHR. *J Biol Chem* 275:36324–36333.
54. Qaid MM, Abdelrahman MM. 2016. Role of insulin and other related hormones in energy metabolism-A review. *Cogent Food Agric* 2:1–18.
55. Petersen MC, Shulman GI. 2018. Mechanisms of Insulin Action and Insulin Resistance. *Physiol Rev* 98:2133–2223.
56. Rhodes CJ, White MF. 2002. Molecular insights into insulin action and secretion. *Eur J Clin Invest* 32:3–13.
57. Sefried S, Häring H-U, Weigert C, Eckstein SS. 2018. Suitability of hepatocyte cell lines HepG2, AML12 and THLE-2 for investigation of insulin signalling and hepatokine gene expression. *Open Biol* 8:1–11.
58. Girard J. 2006. The Inhibitory Effects of Insulin on Hepatic Glucose Production Are Both Direct and Indirect. *Diabetes* 55:S65–S69.
59. Ader M, Bergman RN. 1990. Peripheral effects of insulin dominate suppression of fasting hepatic glucose production. *Am J Physiol Metab* 258:E1020–E1032.
60. Staehr P, Hother-Nielsen O, Beck-Nielsen H. 2004. The Role of the Liver in Type 2 Diabetes. *Rev Endocr Metab Disord* 5:105–110.
61. Edgerton DS, Ramnanan CJ, Grueter CA, Johnson KMS, Lautz M, Neal DW, Williams PE, Cherrington AD. 2009. Effects of Insulin on the Metabolic Control of Hepatic Gluconeogenesis In Vivo. *Diabetes* 58:2766–2775.
62. Cherrington AD, Moore MC, Sindelar DK, Edgerton DS. 2007. Insulin action on the liver in vivo. *Biochem Soc Trans* 35:1171–1174.
63. Fagerholm SA. 2013. Insulin signaling in primary adipocytes in insulin sensitive and insulin resistant states. Linköping University Electronic Press.
64. Danielsson A. 2007. Insulin signalling in human adipocytes – mechanisms of insulin resistance in type 2 diabetes. Linköping University.
65. Saltiel AR, Kahn CR. 2001. Glucose and Lipid Metabolism. *Nature* 414:799–806.
66. Hatting M, Tavares CDJ, Sharabi K, Rines AK, Puigserver P. 2018. Insulin regulation of gluconeogenesis. *Ann N Y Acad Sci* 1411:21–35.
67. Titchenell PM, Lazar MA, Birnbaum MJ. 2017. Unraveling the Regulation of Hepatic Metabolism by Insulin. *Trends Endocrinol Metab* 28:1–9.
68. Taniguchi CM, Emanuelli B, Kahn CR. 2006. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7:85–96.
69. Laychock SG. 2003. Insulin Receptor Signaling, p. 368–380. *In Encyclopedia of*

- Hormones. Elsevier.
70. Saltiel AR, Pessin JE. 2002. Insulin signaling pathways in time and space. *Trends Cell Biol* 12:65–71.
 71. Goldstein BJ, Bittner-Kowalczyk A, White MF, Harbeck M. 2000. Tyrosine Dephosphorylation and Deactivation of Insulin Receptor Substrate-1 by Protein-tyrosine Phosphatase 1B. *J Biol Chem* 275:4283–4289.
 72. Bakke J, Haj FG. 2015. Protein-tyrosine phosphatase 1B substrates and metabolic regulation. *Semin Cell Dev Biol* 37:58–65.
 73. Revuelta-Cervantes J, Mayoral R, Miranda S, González-Rodríguez Á, Fernández M, Martín-Sanz P, Valverde ÁM. 2011. Protein Tyrosine Phosphatase 1B (PTP1B) Deficiency Accelerates Hepatic Regeneration in Mice. *Am J Pathol* 178:1591–1604.
 74. Ravichandran L V, Chen H, Li Y, Quon MJ. 2001. Phosphorylation of PTP1B at Ser 50 by Akt Impairs Its Ability to Dephosphorylate the Insulin Receptor. *Mol Endocrinol* 15:1768–1780.
 75. Delibegovic M, Zimmer D, Kauffman C, Rak K, Hong E-G, Cho Y-R, Kim JK, Kahn BB, Neel BG, Bence KK. 2009. Liver-Specific Deletion of Protein-Tyrosine Phosphatase 1B (PTP1B) Improves Metabolic Syndrome and Attenuates Diet-Induced Endoplasmic Reticulum Stress. *Diabetes* 58:590–599.
 76. Zabolotny JM, Kim Y-B, Welsh LA, Kershaw EE, Neel BG, Kahn BB. 2008. Protein-tyrosine Phosphatase 1B Expression Is Induced by Inflammation in Vivo. *J Biol Chem* 283:14230–14241.
 77. Gum RJ, Gaede LL, Koterski SL, Heindel M, Clampit JE, Zinker BA, Trevillyan JM, Ulrich RG, Jirousek MR, Rondinone CM. 2003. Reduction of protein tyrosine phosphatase 1B increases insulin-dependent signaling in ob/ob mice. *Diabetes* 52:21–28.
 78. Nakashima N, Sharma PM, Imamura T, Bookstein R, Olefsky JM. 2000. The Tumor Suppressor PTEN Negatively Regulates Insulin Signaling in 3T3-L1 Adipocytes. *J Biol Chem* 275:12889–12895.
 79. Simpson L, Li J, Liaw D, Hennessy I, Oliner J, Christians F, Parsons R. 2001. PTEN Expression Causes Feedback Upregulation of Insulin Receptor Substrate 2. *Mol Cell Biol* 21:3947–3958.
 80. Chen C-Y, Chen J, He L, Stiles BL. 2018. PTEN: Tumor Suppressor and Metabolic Regulator. *Front Endocrinol (Lausanne)* 9:1–12.
 81. Filipski E. 2001. Clock checks tumour growthTrends in Molecular Medicine in Molecular Medicine.
 82. Li YZ, Di Cristofano A, Woo M. 2020. Metabolic role of PTEN in insulin signaling and resistance. *Cold Spring Harb Perspect Med* 10:1–17.
 83. Fayard E, Tintignac LA, Baudry A, Hemmings BA. 2005. Protein kinase B/Akt at a glance. *J Cell Sci* 118:5675–5678.
 84. Annibali D, Kriplani LA, Leslie N, Matthews N, Nasi L, Orlic-Milacic, Thorpe F, Zhao M. 2020. Negative regulation of the PI3K/AKT net-work.
 85. Gao T, Furnari F, Newton AC. 2005. PHLPP: A Phosphatase that Directly Dephosphorylates Akt, Promotes Apoptosis, and Suppresses Tumor Growth. *Mol Cell*

- 18:13–24.
86. Kanety H, Feinstein R, Papa MZ, Hemi R, Karasik A. 1995. Tumor Necrosis Factor α -induced Phosphorylation of Insulin Receptor Substrate-1 (IRS-1). *J Biol Chem* 270:23780–23784.
 87. Boura-Halfon S, Zick Y. 2009. Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Metab* 296:E581–E591.
 88. Franke TF. 2008. PI3K/Akt: getting it right matters. *Oncogene* 27:6473–6488.
 89. Morales-Ruiz M, Santel A, Ribera J, Jiménez W. 2017. The Role of Akt in Chronic Liver Disease and Liver Regeneration. *Semin Liver Dis* 37:11–16.
 90. Vanhaesebroeck B, Alessi DR. 2000. The PI3K–PDK1 connection: more than just a road to PKB. *Biochem J* 346:561.
 91. Alessi DR, Cohen P. 1998. Mechanism of activation and function of protein kinase B. *Curr Opin Genet Dev* 8:55–62.
 92. Manning BD, Toker A. 2017. AKT/PKB Signaling: Navigating the Network. *Cell* 169:381–405.
 93. Agarwal AK. 2018. How to explain the AKT phosphorylation of downstream targets in the wake of recent findings. *Proc Natl Acad Sci U S A* 115:E6099–E6100.
 94. Whiteman EL, Cho H, Birnbaum MJ. 2002. Role of Akt/protein kinase B in metabolism. *Trends Endocrinol Metab* 13:444–451.
 95. Alessi DR, Downes CP. 1998. The Role of PI 3-Kinase in Insulin Action. *Biochem Biophys Acta* 1436:151–164.
 96. Colmekci C. 2015. The insulin signalling pathway in skeletal muscle : in silico and in vitro. Eindhoven University of Technology.
 97. Rui L. 2014. Energy Metabolism in the Liver. *Compr Physiol* 4:177–197.
 98. Yabaluri N, Bashyam MD. 2010. Hormonal regulation of gluconeogenic gene transcription in the liver. *J Biosci* 35:473–484.
 99. Barthel A, Schmoll D. 2003. Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Metab* 285:E685–E692.
 100. Lin H V., Accili D. 2011. Hormonal Regulation of Hepatic Glucose Production in Health and Disease. *Cell Metab* 14:9–19.
 101. Carter ME, Brunet A. 2007. FOXO transcription factors. *Curr Biol* 17:R113–R114.
 102. Tzivion G, Dobson M, Ramakrishnan G. 2011. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochim Biophys Acta - Mol Cell Res* 1813:1938–1945.
 103. Tsai W-C, Bhattacharyya N, Han L-Y, Hanover JA, Rechler MM. 2003. Insulin Inhibition of Transcription Stimulated by the Forkhead Protein Foxo1 Is Not Solely due to Nuclear Exclusion. *Endocrinology* 144:5615–5622.
 104. Rines AK, Sharabi K, Tavares CDJ, Puigserver P. 2016. Targeting hepatic glucose metabolism in the treatment of type 2 diabetes. *Nat Rev Drug Discov* 15:786–804.
 105. Leclercq IA, Da Silva Morais A, Schroyen B, Van Hul N, Geerts A. 2007. Insulin resistance in hepatocytes and sinusoidal liver cells: Mechanisms and consequences. *J Hepatol* 47:142–156.

106. Mues C, Zhou J, Manolopoulos KN, Korsten P, Schmoll D, Klotz L-O, Bornstein SR, Klein HH, Barthel A. 2009. Regulation of Glucose-6-Phosphatase Gene Expression by Insulin and Metformin. *Horm Metab Res* 41:730–735.
107. Edgerton DS. 2006. Insulin's direct effects on the liver dominate the control of hepatic glucose production. *J Clin Invest* 116:521–527.
108. Edgerton DS, Kraft G, Smith M, Farmer B, Williams PE, Coate KC, Printz RL, O'Brien RM, Cherrington AD. 2017. Insulin's direct hepatic effect explains the inhibition of glucose production caused by insulin secretion. *JCI Insight* 2:1–14.
109. Vander Kooi BT, Streeper RS, Svitek CA, Oeser JK, Powell DR, O'Brien RM. 2003. The Three Insulin Response Sequences in the Glucose-6-phosphatase Catalytic Subunit Gene Promoter Are Functionally Distinct. *J Biol Chem* 278:11782–11793.
110. Rai LS, Narayanan A, Ray S. 2017. Gluconeogenesis regulation, measurements and disorders, p. 99–113. *In Carbohydrate Metabolism: Theory and Practical Approach*.
111. Zhang X, Yang S, Chen J, Su Z. 2019. Unraveling the Regulation of Hepatic Gluconeogenesis. *Front Endocrinol (Lausanne)* 9:1–17.
112. Grempler R, Kienitz A, Werner T, Meyer M, Barthel A, Ailett F, Sutherland C, Walther R, Schmoll D. 2004. Tumour necrosis factor α decreases glucose-6-phosphatase gene expression by activation of nuclear factor κ B. *Biochem J* 382:471–479.
113. Burchell A. 2001. Von Gierke disease, p. 2120–2122. *In Encyclopedia of Genetics*. Elsevier.
114. Liu X, Wang H, Liang X, Roberts MS. 2017. Hepatic Metabolism in Liver Health and Disease, p. 391–400. *In Liver Pathophysiology: Therapies and Antioxidants*. Elsevier.
115. Jitrapakdee S. 2012. Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis. *Int J Biochem Cell Biol* 44:33–45.
116. Rocha Leão MHM. 2003. Glycogen, p. 2930–2937. *In Encyclopedia of Food Sciences and Nutrition*. Elsevier.
117. Komoda T, Matsunaga T. 2015. Metabolic Pathways in the Human Body, p. 25–63. *In Biochemistry for Medical Professionals*. Elsevier.
118. Ellingwood SS, Cheng A. 2018. Biochemical and clinical aspects of glycogen storage diseases. *J Endocrinol* 238:R131–R141.
119. Woodgett JR, Cohen P. 1984. Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specificity of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochim Biophys Acta (BBA)/Protein Struct Mol* 788:339–347.
120. Blanco A, Blanco G. 2017. Integration and Regulation of Metabolism, p. 425–445. *In Medical Biochemistry*. Elsevier.
121. Henriksen E, Dokken B. 2012. Role of Glycogen Synthase Kinase-3 in Insulin Resistance and Type 2 Diabetes. *Curr Drug Targets* 7:1435–1441.
122. Nikouline SE, Ciaraldi TP, Carter L, Mudaliar S, Park KS, Henry RR. 2001. Impaired muscle glycogen synthase in type 2 diabetes is associated with diminished phosphatidylinositol 3-kinase activation. *J Clin Endocrinol Metab* 86:4307–4314.
123. Henry RR, Ciaraldi TP, Abrams-Carter L, Mudaliar S, Park KS, Nikouline SE. 1996. Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-

- dependent diabetes mellitus subjects: Biochemical and molecular mechanisms. *J Clin Invest* 98:1231–1236.
124. Thorburn AW, Gumbiner B, Bulacan F, Wallace P, Henry RR. 1990. Intracellular glucose oxidation and glycogen synthase activity are reduced in non-insulin-dependent (type II) diabetes independent of impaired glucose uptake. *J Clin Invest* 85:522–529.
 125. Bogardus C, Lillioja S, Stone K, Mott D. 1984. Correlation between muscle glycogen synthase activity and in vivo insulin action in man. *J Clin Invest* 73:1185–1190.
 126. Peraldi P, Spiegelman B. 1998. TNF- α and insulin resistance: Summary and future prospects. *Mol Cell Biochem* 182:169–175.
 127. Onyango AN. 2018. Cellular Stresses and Stress Responses in the Pathogenesis of Insulin Resistance. *Oxid Med Cell Longev*. Hindawi Limited.
 128. DeFronzo RA, Ferrannini E. 1991. Insulin resistance: A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 14:173–194.
 129. Peterson LR. 2006. Obesity and insulin resistance: Effects on cardiac structure, function, and substrate metabolism. *Curr Hypertens Rep* 8:451–456.
 130. Miranda PJ, DeFronzo RA, Califf RM, Guyton JR. 2005. Metabolic syndrome: Definition, pathophysiology, and mechanisms. *Am Heart J* 149:33–45.
 131. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. 1995. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415.
 132. Qatanani M, Lazar MA. 2007. Mechanisms of obesity-associated insulin resistance: many choices on the menu. *Genes Dev* 21:1443–1455.
 133. Marcovecchio ML, Chiarelli F. 2012. The Effects of Acute and Chronic Stress on Diabetes Control. *Sci Signal* 5:1–3.
 134. Hilliard ME, Yi-Frazier JP, Hessler D, Butler AM, Anderson BJ, Jaser S. 2016. Stress and A1c Among People with Diabetes Across the Lifespan. *Curr Diab Rep*. Current Medicine Group LLC 1.
 135. Piroli GG, Grillo CA, Reznikov LR, Adams S, McEwen BS, Charron MJ, Reagan LP. 2007. Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus. *Neuroendocrinology* 85:71–80.
 136. Miguel de Jesus Sousa de Araújo Ribeiro A. 2020. Metabolic Syndrome, p. 47–63. In *Cellular Metabolism and Related Disorders*. IntechOpen.
 137. Kumari R, Kumar S, Kant R. 2019. An update on metabolic syndrome: Metabolic risk markers and adipokines in the development of metabolic syndrome. *Diabetes Metab Syndr Clin Res Rev* 13:2409–2417.
 138. Meshkani R, Adeli K. 2009. Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clin Biochem* 42:1331–1346.
 139. Reaven G. 2004. The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals. *Endocrinol Metab Clin North Am* 33:283–303.
 140. Chung ST, Hsia DS, Chacko SK, Rodriguez LM, Haymond MW. 2015. Increased gluconeogenesis in youth with newly diagnosed type 2 diabetes. *Diabetologia* 58:596–

- 603.
141. Huang X, Liu G, Guo J, Su ZQ. 2018. The PI3K/AKT pathway in obesity and type 2 diabetes. *Int J Biol Sci*. Ivyspring International Publisher.
 142. Leclercq IA, Da Silva Morais A, Schroyen B, Van Hul N, Geerts A. 2007. Insulin resistance in hepatocytes and sinusoidal liver cells: Mechanisms and consequences. *J Hepatol* 47:142–156.
 143. Vergès B. 2015. Pathophysiology of diabetic dyslipidaemia: where are we? *Diabetologia* 58:886–899.
 144. Valverde AM, Burks DJ, Fabregat I, Fisher TL, Carretero J, White MF, Benito M. 2003. Molecular Mechanisms of Insulin Resistance in IRS-2-Deficient Hepatocytes. *Diabetes*.
 145. Hotamisligil GS, Spiegelman BM. 1994. Perspectives in Diabetes Tumor Necrosis Factor α : A Key Component of the Obesity-Diabetes Link. *Diabetes*.
 146. Hotamisligil GS. 1999. The role of TNF α and TNF receptors in obesity and insulin resistance. *J Intern Med* 245:621–625.
 147. Solinas G, Becattini B. 2017. JNK at the crossroad of obesity, insulin resistance, and cell stress response. *Mol Metab* 6:174–184.
 148. Borst SE. 2004. The Role of TNF- α in Insulin Resistance. *Endocrine* 23:177–182.
 149. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. 1996. IRS-1-Mediated Inhibition of Insulin Receptor Tyrosine Kinase Activity in TNF-alpha- and Obesity-Induced Insulin Resistance. *Science* (80-) 271:665–670.
 150. Del Aguila LF, Claffey KP, Kirwan JP. 1999. TNF- α impairs insulin signaling and insulin stimulation of glucose uptake in C2C12 muscle cells. *Am J Physiol - Endocrinol Metab* 276.
 151. Egawa K, Maegawa H, Shimizu S, Morino K, Nishio Y, Bryer-Ash M, Cheung AT, Kolls JK, Kikkawa R, Kashiwagi A. 2001. Protein-tyrosine Phosphatase-1B Negatively Regulates Insulin Signaling in L6 Myocytes and Fao Hepatoma Cells. *J Biol Chem* 276:10207–10211.
 152. Klamann LD, Boss O, Peroni OD, Kim JK, Martino JL, Zabolotny JM, Moghal N, Lubkin M, Kim Y-B, Sharpe AH, Stricker-Krongrad A, Shulman GI, Neel BG, Kahn BB. 2000. Increased Energy Expenditure, Decreased Adiposity, and Tissue-Specific Insulin Sensitivity in Protein-Tyrosine Phosphatase 1B-Deficient Mice. *Mol Cell Biol* 20:5479–5489.
 153. Chen PJ, Cai SP, Huang C, Meng XM, Li J. 2015. Protein tyrosine phosphatase 1B (PTP1B): A key regulator and therapeutic target in liver diseases. *Toxicology*. Elsevier Ireland Ltd.
 154. Juryneac MJ, Grunwald DJ. 2010. SHIP2, a factor associated with diet-induced obesity and insulin sensitivity, attenuates FGF signaling in vivo. *Dis Model Mech* 3:733–742.
 155. Abdelsalam, Korashy, Zeidan, Agouni. 2019. The Role of Protein Tyrosine Phosphatase (PTP)-1B in Cardiovascular Disease and Its Interplay with Insulin Resistance. *Biomolecules* 9:286.
 156. Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA KC. 2000. Loss of insulin signaling in hepatocytes leads to severe insulin resistance. *Mol Cell* 6:87–97.

157. Kubota N, Kubota T, Itoh S, Kumagai H, Kozono H, Takamoto I, Mineyama T, Ogata H, Tokuyama K, Ohsugi M, Sasako T, Moroi M, Sugi K, Kakuta S, Iwakura Y, Noda T, Ohnishi S, Nagai R, Tobe K, Terauchi Y, Ueki K, Kadokawa T. 2008. Dynamic Functional Relay between Insulin Receptor Substrate 1 and 2 in Hepatic Insulin Signaling during Fasting and Feeding. *Cell Metab* 8:49–64.
158. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900–904.
159. Winnay JN, Solheim MH, Dirice E, Sakaguchi M, Noh HL, Kang HJ, Takahashi H, Chudasama KK, Kim JK, Molven A, Kahn CR, Njølstad PR. 2016. PI3-kinase mutation linked to insulin and growth factor resistance in vivo. *J Clin Invest* 126:1401–1412.
160. Rotter Sopasakis V, Chaudhari A, Ejeskär K, Wettergren Y, Kahn CR. 2018. Hepatic deletion of p110 α and p85 α results in insulin resistance despite sustained IRS1-associated phosphatidylinositol kinase activity [version 2; referees: 1 approved, 2 approved with reservations]. *F1000Research* 6:1–24.
161. Saad MJA, Folli F, Kahn CR. 1995. Insulin and dexamethasone regulate insulin receptors, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in fao hepatoma cells. *Endocrinology* 136:1579–1588.
162. De Alvaro C, Teruel T, Hernandez R, Lorenzo M. 2004. Tumor Necrosis Factor α Produces Insulin Resistance in Skeletal Muscle by Activation of Inhibitor κ B Kinase in a p38 MAPK-dependent Manner. *J Biol Chem* 279:17070–17078.
163. Schmidt-Arras D, Rose-John S. 2016. IL-6 pathway in the liver: From physiopathology to therapy. *J Hepatol* 64:1403–1415.
164. Ueki K, Kondo T, Kahn CR. 2004. Suppressor of Cytokine Signaling 1 (SOCS-1) and SOCS-3 Cause Insulin Resistance through Inhibition of Tyrosine Phosphorylation of Insulin Receptor Substrate Proteins by Discrete Mechanisms. *Mol Cell Biol* 24:5434–5446.
165. Jorgensen SB, O'Neill HM, Sylow L, Honeyman J, Hewitt KA, Palanivel R, Fullerton MD, Öberg L, Balandran A, Galic S, Van Der Poel C, Trounce IA, Lynch GS, Schertzer JD, Steinberg GR. 2013. Deletion of skeletal muscle SOCS3 prevents insulin resistance in obesity. *Diabetes* 62:56–64.
166. Mashili F, Chibalin A V., Krook A, Zierath JR. 2013. Constitutive STAT3 phosphorylation contributes to skeletal muscle insulin resistance in type 2 diabetes. *Diabetes* 62:457–465.
167. Dou L, Zhao T, Wang L, Huang X, Jiao J, Gao D, Zhang H, Shen T, Man Y, Wang S, Li J. 2013. MiR-200s contribute to interleukin-6 (IL-6)-induced insulin resistance in hepatocytes. *J Biol Chem* 288:22596–22606.
168. Black PH. 2002. Stress and the inflammatory response: A review of neurogenic inflammation. *Brain Behav Immun*.
169. Miller GE, Cohen S, Ritchey AK. 2002. Chronic psychological stress and the regulation of pro-inflammatory cytokines: A glucocorticoid-resistance model. *Heal Psychol* 21:531–541.
170. Liang ZQ, Huang LZ, Qu JF, Zhao MW. 2018. Association between endogenous cortisol level and the risk of central serous chorioretinopathy: a Meta-analysis. *Int J*

- Ophthalmol 11:296–300.
171. Miller WL, Auchus RJ. 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev* 32:81–151.
 172. Timmermans S, Souffriau J, Libert C. 2019. A General Introduction to Glucocorticoid Biology. *Front Immunol* 10.
 173. Miller WL. 2018. The Hypothalamic-Pituitary-Adrenal Axis: A Brief History. *Horm Res Paediatr*. S. Karger AG.
 174. Gjerstad JK, Lightman SL, Spiga F. 2018. Role of glucocorticoid negative feedback in the regulation of HPA axis pulsatility. *Stress* 21:403–416.
 175. Stephens MAC, McCaul ME, Wand GS. 2014. The Potential Role of Glucocorticoids and the HPA Axis in Alcohol Dependence, p. 429–450. *In Neurobiology of Alcohol Dependence*. Elsevier.
 176. Charmandari E, Tsigos C, Chrousos G. 2005. Endocrinology of the stress response. *Annu Rev Physiol* 67:259–284.
 177. Dineen R, Stewart PM, Sherlock M. 2019. Factors impacting on the action of glucocorticoids in patients receiving glucocorticoid therapy. *Clin Endocrinol (Oxf)* 90:3–14.
 178. Wang M. 2005. The role of glucocorticoid action in the pathophysiology of the Metabolic Syndrome. *Nutr Metab* 2.
 179. Ramamoorthy S, Cidlowski JA. 2016. Corticosteroids-Mechanisms of Action in Health and Disease. *Rheum Dis Clin North Am* 42:15–31.
 180. Kewalramani G, Puthanveetil P, Min SK, Wang F, Lee V, Hau N, Beheshti E, Ng N, Abrahani A, Rodrigues B. 2008. Acute dexamethasone-induced increase in cardiac lipoprotein lipase requires activation of both Akt and stress kinases. *Am J Physiol - Endocrinol Metab* 295:137–147.
 181. Schäcke H, Döcke WD, Asadullah K. 2002. Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther*.
 182. Paragliola RM, Papi G, Pontecorvi A, Corsello SM. 2017. Treatment with Synthetic Glucocorticoids and the Hypothalamus-Pituitary-Adrenal Axis. *Int J Mol Sci* 18:2201.
 183. Adcock IM, Mumby S. 2016. Glucocorticoids, p. 171–196. *In Handbook of Experimental Pharmacology*. Springer New York LLC.
 184. Czock D, Keller F, Rasche FM, Haussler U. 2005. Pharmacokinetics and Pharmacodynamics of Systemically Administered Glucocorticoids. *Clin Pharmacokinet* 44:61–98.
 185. Gensler LS. 2013. Glucocorticoids: Complications to Anticipate and Prevent. *The Neurohospitalist* 3:92–97.
 186. Niu L, Chen Q, Hua C, Geng Y, Cai L, Tao S, Ni Y, Zhao R. 2018. Effects of chronic dexamethasone administration on hyperglycemia and insulin release in goats. *J Anim Sci Biotechnol* 9:1–10.
 187. Stahn C, Buttigereit F. 2008. Genomic and nongenomic effects of glucocorticoids. *Nat Clin Pract Rheumatol* 4:525–533.
 188. Schaaf MJM, Cidlowski JA. 2002. Molecular mechanisms of glucocorticoid action and

- resistance. *J Steroid Biochem Mol Biol* 83:37–48.
- 189. Schoneveld OJLM, Gaemers IC, Lamers WH. 2004. Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta - Gene Struct Expr* 1680:114–128.
 - 190. Radoja N, Komine M, Jho SH, Blumenberg M, Tomic-Canic M. 2000. Novel Mechanism of Steroid Action in Skin through Glucocorticoid Receptor Monomers. *Mol Cell Biol* 20:4328–4339.
 - 191. Hudson WH, Youn C, Ortlund EA. 2013. The structural basis of direct glucocorticoid-mediated transrepression. *Nat Struct Mol Biol* 20:53–58.
 - 192. Surjit M, Ganti KP, Mukherji A, Ye T, Hua G, Metzger D, Li M, Chambon P. 2011. Widespread negative response elements mediate direct repression by agonist-ligated glucocorticoid receptor. *Cell* 145:224–241.
 - 193. Escoter-Torres L, Caratti G, Mechtidou A, Tuckermann J, Uhlenhaut NH, Vettorazzi S. 2019. Fighting the Fire: Mechanisms of Inflammatory Gene Regulation by the Glucocorticoid Receptor. *Front Immunol* 10.
 - 194. Malkoski SP, Dorin RI. 1999. Composite glucocorticoid regulation at a functionally defined negative glucocorticoid response element of the human corticotropin-releasing hormone gene. *Mol Endocrinol* 13:1629–1644.
 - 195. Vandevyver S, Dejager L, Tuckermann J, Libert C. 2013. New Insights into the Anti-inflammatory Mechanisms of Glucocorticoids: An Emerging Role for Glucocorticoid-Receptor-Mediated Transactivation. *Endocrinology* 154:993–1007.
 - 196. Schiller BJ, Chodankar R, Watson LC, Stallcup MR, Yamamoto KR. 2014. Glucocorticoid receptor binds half sites as a monomer and regulates specific target genes. *Genome Biol* 15.
 - 197. Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR. 1990. Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. *Science* (80-) 249:1266–1272.
 - 198. Lim HW, Uhlenhaut NH, Rauch A, Weiner J, Hübner S, Hübner N, Won KJ, Lazar MA, Tuckermann J, Steger DJ. 2015. Genomic redistribution of GR monomers and dimers mediates transcriptional response to exogenous glucocorticoid in vivo. *Genome Res* 25:836–844.
 - 199. Cruz-Topete D, Cidlowski JA. 2015. One Hormone, Two Actions: Anti- and Pro-Inflammatory Effects of Glucocorticoids. *Neuroimmunomodulation* 22:20–32.
 - 200. Luecke HF, Yamamoto KR. 2005. The glucocorticoid receptor blocks P-TEFb recruitment by NFκB to effect promoter-specific transcriptional repression. *Genes Dev* 19:1116–1127.
 - 201. Rao NAS, McCalman MT, Moulos P, Francois KJ, Chatziloannou A, Kolisis FN, Alexis MN, Mitsiou DJ, Stunnenberg HG. 2011. Coactivation of GR and NFKB alters the repertoire of their binding sites and target genes. *Genome Res* 21:1404–1416.
 - 202. Petta I, Dejager L, Ballegeer M, Lievens S, Tavernier J, De Bosscher K, Libert C. 2016. The Interactome of the Glucocorticoid Receptor and Its Influence on the Actions of Glucocorticoids in Combatting Inflammatory and Infectious Diseases. *Microbiol Mol Biol Rev* 80:495–522.
 - 203. Barnes PJ. 1998. Anti-inflammatory actions of glucocorticoids: Molecular mechanisms. *Clin Sci* 94:557–572.

204. Liberman AC, Budziński ML, Sokn C, Gobbini RP, Steininger A, Arzt E. 2018. Regulatory and mechanistic actions of glucocorticoids on T and inflammatory cells. *Front Endocrinol (Lausanne)*. Frontiers Media S.A.
205. Herrlich P. 2001. Cross-talk between glucocorticoid receptor and AP-1. *Oncogene* 20:2465–2475.
206. Hudson WH, Vera IMS de, Nwachukwu JC, Weikum ER, Herbst AG, Yang Q, Bain DL, Nettles KW, Kojetin DJ, Ortlund EA. 2018. Cryptic glucocorticoid receptor-binding sites pervade genomic NF- κ B response elements. *Nat Commun* 9:1337.
207. Pasieka AM, Rafacho A. 2016. Impact of glucocorticoid excess on glucose tolerance: Clinical and preclinical evidence. *Metabolites* 6.
208. Yan Y-X, Xiao H-B, Wang S-S, Zhao J, He Y, Wang W, Dong J. 2016. Investigation of the Relationship Between Chronic Stress and Insulin Resistance in a Chinese Population. *J Epidemiol* 26:355–360.
209. Lu Y, Xiong X, Wang X, Zhang Z, Li J, Shi G, Yang J, Zhang H, Ning G, Li X. 2013. Yin Yang 1 promotes hepatic gluconeogenesis through upregulation of glucocorticoid receptor. *Diabetes* 62:1064–1073.
210. Shi Y, Qiao J, Mu B, Zuo B, Yuan J. 2017. 3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) ameliorated dexamethasone induced hepatic gluconeogenesis through activation of Akt/FoxO1 pathway. *Biochem Biophys Res Commun* 493:286–290.
211. Kuo T, McQueen A, Chen TC, Wang JC. 2015. Regulation of glucose homeostasis by glucocorticoids. *Adv Exp Med Biol* 872:99–126.
212. Lin B, Morris DW, Chou JY. 1998. Hepatocyte nuclear factor 1 α is an accessory factor required for activation of glucose-6-phosphatase gene transcription by glucocorticoids. *DNA Cell Biol* 17:967–974.
213. El-Sonbaty YA, Suddek GM, Megahed N, Gameil NM. 2019. Protocatechuic acid exhibits hepatoprotective, vasculoprotective, antioxidant and insulin-like effects in dexamethasone-induced insulin-resistant rats. *Biochimie* 167:119–134.
214. Vander Kooi BT, Onuma H, Oeser JK, Svitek CA, Allen SR, Vander Kooi CW, Chazin WJ, O'Brien RM. 2005. The glucose-6-phosphatase catalytic subunit gene promoter contains both positive and negative glucocorticoid response elements. *Mol Endocrinol* 19:3001–3022.
215. Zhang Y, Guan Q, Liu Y, Zhang Y, Chen Y, Chen J, Liu Y, Su Z. 2018. Regulation of hepatic gluconeogenesis by nuclear factor Y transcription factor in mice. *J Biol Chem* 293:7894–7904.
216. Hanson RW, Reshef L. 1997. Regulation Of Phosphoenolpyruvate Carboxykinase (GTP) Gene Expression. *Annu Rev Biochem* 66:581–611.
217. Shao J, Qiao L, Janssen RC, Pagliassotti M, Friedman JE. 2005. Chronic hyperglycemia enhances PEPCK gene expression and hepatocellular glucose production via elevated liver activating protein/liver inhibitory protein ratio. *Diabetes* 54:976–984.
218. Andrade MVM, Hiragun T, Beaven MA. 2004. Dexamethasone Suppresses Antigen-Induced Activation of Phosphatidylinositol 3-Kinase and Downstream Responses in Mast Cells. *J Immunol* 172:7254–7262.
219. Kim J, Park MY, Kim HK, Park Y, Whang KY. 2016. Cortisone and dexamethasone

- inhibit myogenesis by modulating the AKT/mTOR signaling pathway in C2C12. *Biosci Biotechnol Biochem* 80:2093–2099.
220. Saad MJA, Folli F, Kahn JA, Kahn CR. 1993. Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *J Clin Invest* 92:2065–2072.
221. Almon RR, DuBois DC, Jin JY, Jusko WJ. 2005. Temporal profiling of the transcriptional basis for the development of corticosteroid-induced insulin resistance in rat muscle. *J Endocrinol* 184:219–232.
222. Turnbow MA, Keller SR, Rice KM, Garner CW. 1994. Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3-L1 adipocytes. *J Biol Chem* 269:2516–2520.
223. Giorgino F, Almahfouz A, Goodyear LJ, Smith RJ. 1993. Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. *J Clin Invest* 91:2020–2030.
224. Miao H, Zhang Y, Lu Z, Liu Q, Gan L. 2012. FOXO1 involvement in insulin resistance-related pro-inflammatory cytokine production in hepatocytes. *Inflamm Res* 61:349–358.
225. Bastard J, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B. 2006. Recent advances in the relationship between obesity. *Eur Cytokine Netw* 17:4–12.
226. Monteiro R, Azevedo I. 2010. Chronic Inflammation in Obesity and the Metabolic Syndrome. *Mediators Inflamm* 2010:1–10.
227. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao L. 2018. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* 9:7204–7218.
228. Zelová H, Hošek J. 2013. TNF- α signalling and inflammation: interactions between old acquaintances. *Inflamm Res* 62:641–651.
229. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830.
230. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. 2003. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808.
231. Luft VC, Schmidt MI, Pankow JS, Couper D, Ballantyne CM, Young JH, Duncan BB. 2013. Chronic inflammation role in the obesity-diabetes association: a case-cohort study. *Diabetol Metab Syndr* 5:31.
232. van Greevenbroek MMJ, Schalkwijk CG, Stehouwer CDA. 2013. Obesity-associated low-grade inflammation in type 2 diabetes mellitus: Causes and consequences. *Neth J Med* 71:174–187.
233. Feghali CA, Wright TM. 1997. Cytokines in acute and chronic inflammation. *Front Biosci* 2:12–26.
234. Zhang J-M, An J. 2007. Cytokines, Inflammation, and Pain. *Int Anesthesiol Clin* 45:27–37.
235. Dinarello CA. 2000. Proinflammatory Cytokines. *Chest* 118:503–508.
236. Weichhaus M, Broom I, Bermano G. 2011. The molecular contribution of TNF- α in the

- link between obesity and breast cancer. *Oncol Rep* 25:477–483.
237. Wullaert A, van Loo G, Heyninck K, Beyaert R. 2007. Hepatic Tumor Necrosis Factor Signaling and Nuclear Factor- κ B: Effects on Liver Homeostasis and Beyond. *Endocr Rev* 28:365–386.
238. Libermann TA, Baltimore D. 1990. Activation of interleukin-6 gene expression through the NF- κ B transcription factor. *Mol Cell Biol* 10:2327–2334.
239. Son Y-H, Jeong Y-T, Lee K-A, Choi K-H, Kim S-M, Rhim B-Y, Kim K. 2008. Roles of MAPK and NF- κ B in Interleukin-6 Induction by Lipopolysaccharide in Vascular Smooth Muscle Cells. *J Cardiovasc Pharmacol* 51:71–77.
240. Agrawal A, Samols D, Kushner I. 2003. Transcription factor c-Rel enhances C-reactive protein expression by facilitating the binding of C/EBP β to the promoter. *Mol Immunol* 40:373–380.
241. Edbrooke MR, Foldi J, Cheshire JK, Li F, Faulkes DJ, Woo P. 1991. Constitutive and NF- κ B—like proteins in the regulation of the serum amyloid a gene by interleukin 1. *Cytokine* 3:380–388.
242. Li X, Liao WSL. 1991. Expression of rat serum amyloid A1 gene involves both C/EBP-like and NF κ B-like transcription factors. *J Biol Chem* 266:15192–15201.
243. Hou B, Eren M, Painter CA, Covington JW, Dixon JD, Schoenhard JA, Vaughan DE. 2004. Tumor Necrosis Factor α Activates the Human Plasminogen Activator Inhibitor-1 Gene through a Distal Nuclear Factor κ B Site. *J Biol Chem* 279:18127–18136.
244. Thorn CF, Lu ZY, Whitehead AS. 2004. Regulation of the Human Acute Phase Serum Amyloid A Genes by Tumour Necrosis Factor- α , Interleukin-6 and Glucocorticoids in Hepatic and Epithelial Cell Lines. *Scand J Immunol* 59:152–158.
245. Son DS, Roby KF, Terranova PF. 2004. Tumor necrosis factor- α induces serum amyloid A3 in mouse granulosa cells. *Endocrinology* 145:2245–2252.
246. Xu C, Chakravarty K, Kong X, Tuy TT, Arinze IJ, Bone F, Massillon D. 2007. Several transcription factors are recruited to the glucose-6-phosphatase gene promoter in response to palmitate in rat hepatocytes and H4IIE cells. *J Nutr* 137:554–559.
247. Kostadinova RM, Nawrocki AR, Frey FJ, Frey BM. 2005. Tumor necrosis factor alpha and phorbol 12-myristate-13-acetate down-regulate human 11 β -hydroxysteroid dehydrogenase type 2 through p50/p50 NF- κ B homodimers and Egr-1. *FASEB J* 19:1–30.
248. Black PH. 2006. The inflammatory consequences of psychologic stress: Relationship to insulin resistance, obesity, atherosclerosis and diabetes mellitus, type II. *Med Hypotheses* 67:879–891.
249. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. 2001. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *J Am Med Assoc* 286:327–334.
250. Reeh H, Rudolph N, Billing U, Christen H, Streif S, Bullinger E, Schliemann-Bullinger M, Findeisen R, Schaper F, Huber HJ, Dittrich A. 2019. Response to IL-6 trans- A nd IL-6 classic signalling is determined by the ratio of the IL-6 receptor α to gp130 expression: Fusing experimental insights and dynamic modelling. *Cell Commun Signal* 17.
251. Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L. 1998. Interleukin-6-

- type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* 334:297–314.
252. Costa-Pereira AP. 2014. Regulation of IL-6-type cytokine responses by MAPKs. *Biochem Soc Trans* 42:59–62.
253. Pocai A, Obici S, Schwartz GJ, Rossetti L. 2005. A brain-liver circuit regulates glucose homeostasis. *Cell Metab* 1:53–61.
254. Rotter V, Nagaev I, Smith U. 2003. Interleukin-6 (IL-6) Induces Insulin Resistance in 3T3-L1 Adipocytes and Is, Like IL-8 and Tumor Necrosis Factor- α , Overexpressed in Human Fat Cells from Insulin-resistant Subjects. *J Biol Chem* 278:45777–45784.
255. Medina EA, Afsari RR, Ravid T, Sianna Castillo S, Erickson KL, Goldkorn T. 2005. Tumor necrosis factor- α decreases Akt protein levels in 3T3-L1 adipocytes via the caspase-dependent ubiquitination of Akt. *Endocrinology* 146:2726–2735.
256. Wunderlich FT, Ströhle P, Könner AC, Gruber S, Tovar S, Brönneke HS, Juntti-Berggren L, Li LS, Van Rooijen N, Libert C, Berggren PO, Brüning JC. 2010. Interleukin-6 signaling in liver-parenchymal cells suppresses hepatic inflammation and improves systemic insulin action. *Cell Metab* 12:237–249.
257. Inoue H, Ogawa W, Ozaki M, Haga S, Matsumoto M, Furukawa K, Hashimoto N, Kido Y, Mori T, Sakaue H, Teshigawara K, Jin S, Iguchi H, Hiramatsu R, LeRoith D, Takeda K, Akira S, Kasuga M. 2004. Role of STAT-3 in regulation of hepatic gluconeogenic genes and carbohydrate metabolism in vivo. *Nat Med* 10:168–174.
258. Ramadoss P, Unger-Smith NE, Lam FS, Hollenberg AN. 2009. STAT3 targets the regulatory regions of gluconeogenic genes in vivo. *Mol Endocrinol* 23:827–837.
259. Thorn CF, Whitehead AS. 2002. Differential Glucocorticoid Enhancement of the Cytokine-Driven Transcriptional Activation of the Human Acute Phase Serum Amyloid A Genes, SAA1 and SAA2. *J Immunol* 169:399–406.
260. Visser K, Smith C, Louw A. 2010. Interplay of the inflammatory and stress systems in a hepatic cell line: Interactions between glucocorticoid receptor agonists and interleukin-6. *Endocrinology* 151:5279–5293.
261. Liu QS, Nilsen-Hamilton M, Xiong SD. 2003. Synergistic regulation of the acute phase protein SIP24/24p3 by glucocorticoid and pro-inflammatory cytokines. *Acta Physiol Sin* 55:525–529.
262. Depraetere S, Willems J, Joniau M. 1991. Stimulation of CRP secretion in HepG2 cells: Cooperative effect of dexamethasone and interleukin 6. *Agents Actions* 34:369–375.
263. Fasshauer M, Klein J, Kralisch S, Klier M, Lossner U, Bluher M, Paschke R. 2004. Serum amyloid A3 expression is stimulated by dexamethasone and interleukin-6 in 3T3-L1 adipocytes. *J Endocrinol* 183:561–567.
264. Speelman T. 2020. The effect of acute phase proteins on hepatic insulin signalling. Stellenbosch University.
265. Robertson S, Allie-Reid F, Vanden Berghe W, Visser K, Binder A, Africander D, Vismer M, De Bosscher K, Hapgood J, Haegeman G, Louw A. 2010. Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of compound A. *J Biol Chem* 285:8061–8075.
266. Verhoog N, Allie-Reid F, Berghe W, Vanden, Smith C, Haegeman G, Hapgood J, Louw A. 2014. Inhibition of corticosteroid-binding globulin gene expression by

- glucocorticoids involves C/EBP β . PLoS One 9:1–14.
267. Aranda PS, Lajoie DM, Jorcyk CL. 2012. Bleach gel: A simple agarose gel for analyzing RNA quality. Electrophoresis 33:366–369.
268. Chen L, Chen R, Wang H, Liang F. 2015. Mechanisms Linking Inflammation to Insulin Resistance. Int J Endocrinol 2015:1–9.
269. Kamba A, Daimon M, Murakami H, Otaka H, Matsuki K, Sato E, Tanabe J, Takayasu S, Matsuhashi Y, Yanagimachi M, Terui K, Kageyama K, Tokuda I, Takahashi I, Nakaji S. 2016. Association between higher serum cortisol levels and decreased insulin secretion in a general population. PLoS One 11.
270. Petersen KF, Shulman GI. 2006. Etiology of Insulin Resistance. Am J Med 119:S10–S16.
271. Nicolau J, Lequerré T, Bacquet H, Vittecoq O. 2017. Rheumatoid arthritis, insulin resistance, and diabetes. Jt Bone Spine 84:411–416.
272. Hua C, Buttgereit F, Combe B. 2020. Glucocorticoids in rheumatoid arthritis: current status and future studies. RMD Open 6:e000536.
273. Den Uyl D, Van Raalte DH, Nurmohamed MT, Lems WF, Bijlsma JWJ, Hoes JN, Dijkmans BAC, Diamant M. 2012. Metabolic effects of high-dose prednisolone treatment in early rheumatoid arthritis: Balance between diabetogenic effects and inflammation reduction. Arthritis Rheum 64:639–646.
274. Paolino S, Cutolo M, Pizzorni C. 2017. Glucocorticoid management in rheumatoid arthritis: morning or night low dose? Reumatologia/Rheumatology 4:189–197.
275. Moreland LW, O'Dell JR. 2002. Glucocorticoids and rheumatoid arthritis: Back to the future? Arthritis Rheum 46:2553–2563.
276. Landa-Galvan H V., Rios-Castro E, Romero-Garcia T, Rueda A, Olivares-Reyes JA. 2020. Metabolic syndrome diminishes insulin-induced Akt activation and causes a redistribution of Akt-interacting proteins in cardiomyocytes. PLoS One 15:e0228115.
277. Nelson BA, Robinson KA, Buse MG. 2002. Defective Akt activation is associated with glucose- but not glucosamine-induced insulin resistance. Am J Physiol Metab 282:E497–E506.
278. Hart JR, Vogt PK. 2011. Phosphorylation of AKT: A mutational analysis. Oncotarget 2:467–476.
279. Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, Dedhar S. 2001. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: Critical roles for kinase activity and amino acids arginine 211 and serine 343. J Biol Chem 276:27462–27469.
280. Patel S, Doble BW, MacAulay K, Sinclair EM, Drucker DJ, Woodgett JR. 2008. Tissue-Specific Role of Glycogen Synthase Kinase 3 β in Glucose Homeostasis and Insulin Action. Mol Cell Biol 28:6314–6328.
281. Beurel E, Grieco SF, Jope RS. 2015. Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. Pharmacol Ther 148:114–131.
282. Frame S, Cohen P. 2001. GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359:1–16.
283. van Schaftingen E, Gerin I. 2002. The glucose-6-phosphatase system. Biochem J

- 362:513–532.
284. Al-Lawati JA. 2017. Diabetes Mellitus: A Local and Global Public Health Emergency! *Oman Med J* 32:177–179.
285. Wang C, Chen Z, Li S, Zhang Y, Jia S, Li J, Chi Y, Miao Y, Guan Y, Yang J. 2014. Hepatic overexpression of ATP synthase β subunit activates PI3K/Akt pathway to ameliorate hyperglycemia of diabetic mice. *Diabetes* 63:947–959.
286. Biddinger SB, Kahn CR. 2006. From Mice To Men: Insights into the Insulin Resistance Syndromes. *Annu Rev Physiol* 68:123–158.
287. Shaw JE, Sicree RA, Zimmet PZ. 2010. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract*.
288. Kim O-K, Jun W, Lee J. 2015. Mechanism of ER Stress and Inflammation for Hepatic Insulin Resistance in Obesity. *Ann Nutr Metab* 67:218–227.
289. Yecies JL, Zhang HH, Menon S, Liu S, Yecies D, Lipovsky AI, Gorgun C, Kwiatkowski DJ, Hotamisligil GS, Lee CH, Manning BD. 2011. Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell Metab* 14:21–32.
290. Xing Y, Zhang J, Wei H, Zhang H, Guan Y, Wang X, Tong X. 2019. Reduction of the PI3K/Akt related signaling activities in skeletal muscle tissues involves insulin resistance in intrauterine growth restriction rats with catch-up growth. *PLoS One* 14.
291. Osawa Y, Nagaki M, Banno Y, Brenner DA, Asano T, Nozawa Y, Moriwaki H, Nakashima S. 2002. Tumor necrosis factor alpha-induced interleukin-8 production via NF- κ B and phosphatidylinositol 3-kinase/Akt pathways inhibits cell apoptosis in human hepatocytes. *Infect Immun* 70:6294–6301.
292. Kim J-H, Kim JE, Liu H-Y, Cao W, Chen J. 2008. Regulation of Interleukin-6-induced Hepatic Insulin Resistance by Mammalian Target of Rapamycin through the STAT3-SOCS3 Pathway. *J Biol Chem* 283:708–715.
293. Gomez-Sanchez E, Gomez-Sanchez CE. 2014. The multifaceted mineralocorticoid receptor. *Compr Physiol* 4:965–994.
294. Ruzzin J, Wagman AS, Jensen J. 2005. Glucocorticoid-induced insulin resistance in skeletal muscles: Defects in insulin signalling and the effects of a selective glycogen synthase kinase-3 inhibitor. *Diabetologia* 48:2119–2130.
295. Kuo Y-C, Huang K-Y, Yang C-H, Yang Y-S, Lee W-Y, Chiang C-W. 2008. Regulation of Phosphorylation of Thr-308 of Akt, Cell Proliferation, and Survival by the B55 α Regulatory Subunit Targeting of the Protein Phosphatase 2A Holoenzyme to Akt. *J Biol Chem* 283:1882–1892.
296. Wei Y, Zhou J, Yu H, Jin X. 2019. AKT phosphorylation sites of Ser473 and Thr308 regulate AKT degradation. *Biosci Biotechnol Biochem* 83:429–435.
297. Mansley MK, Watt GB, Francis SL, Walker DJ, Land SC, Bailey MA, Wilson SM. 2016. Dexamethasone and insulin activate serum and glucocorticoid-inducible kinase 1 (SGK1) via different molecular mechanisms in cortical collecting duct cells. *Physiol Rep* 4:e12792.
298. Sandra F, Matsuki NA, Takeuchi H, Ikebe T, Kanematsu T, Ohishi M, Hirata M. 2002. TNF inhibited the apoptosis by activation of Akt serine/threonine kinase in the human head and neck squamous cell carcinoma. *Cell Signal* 14:771–778.

299. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* 15:6541–6551.
300. Yang J, Cron P, Good VM, Thompson V, Hemmings BA, Barford D. 2002. Crystal structure of an activated Akt/Protein Kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol* 9:940–944.
301. Zegeye MM, Lindkvist M, Fälker K, Kumawat AK, Paramel G, Grenegård M, Sirsjö A, Ljungberg LU. 2018. Activation of the JAK/STAT3 and PI3K/AKT pathways are crucial for IL-6 trans-signaling-mediated pro-inflammatory response in human vascular endothelial cells. *Cell Commun Signal* 16:55–65.
302. Liberman Z, Eldar-Finkelman H. 2005. Serine 332 Phosphorylation of Insulin Receptor Substrate-1 by Glycogen Synthase Kinase-3 Attenuates Insulin Signaling. *J Biol Chem* 280:4422–4428.
303. MacAulay K, Doble BW, Patel S, Hansotia T, Sinclair EM, Drucker DJ, Nagy A, Woodgett JR. 2007. Glycogen Synthase Kinase 3 α -Specific Regulation of Murine Hepatic Glycogen Metabolism. *Cell Metab* 6:329–337.
304. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. 2000. Requirement for glycogen synthase kinase-3 β in cell survival and NF- κ B activation. *Nature* 406:86–90.
305. Wang H, Fang R, Wang X-F, Zhang F, Chen D-Y, Zhou B, Wang H-S, Cai S-H, Du J. 2013. Stabilization of Snail through AKT/GSK-3 β signaling pathway is required for TNF- α -induced epithelial–mesenchymal transition in prostate cancer PC3 cells. *Eur J Pharmacol* 714:48–55.
306. Jope RS, J YCJ, Eléonore B. 2007. Glycogen Synthase Kinase-3 (GSK3): Inflammation, Diseases, and Therapeutics. *Neurochem Res* 32:577–595.
307. Medina M, Wandosell F. 2011. Deconstructing GSK-3: The Fine Regulation of Its Activity. *Int J Alzheimers Dis* 2011:1–12.
308. Eldar-Finkelman H, Krebs EG. 1997. Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc Natl Acad Sci* 94:9660–9664.
309. Linding R, Jensen LJ, Osthimer GJ, van Vugt MATM, Jørgensen C, Miron IM, Diella F, Colwill K, Taylor L, Elder K, Metalnikov P, Nguyen V, Pascalescu A, Jin J, Park JG, Samson LD, Woodgett JR, Russell RBB, Bork P, Yaffe MB, Pawson T. 2007. Systematic Discovery of In Vivo Phosphorylation Networks. *Cell* 129:1415–1426.
310. Hartigan JA, Xiong W-C, Johnson GVW. 2001. Glycogen Synthase Kinase 3 β Is Tyrosine Phosphorylated by PYK2. *Biochem Biophys Res Commun* 284:485–489.
311. Kim L, Liu J, Kimmel AR. 1999. The Novel Tyrosine Kinase ZAK1 Activates GSK3 to Direct Cell Fate Specification. *Cell* 99:399–408.
312. Argaud D, Zhang Q, Pan W, Maitra S, Pilkis SJ, Lange AJ. 1996. Regulation of rat liver glucose-6-phosphatase gene expression in different nutritional and hormonal states: Gene structure and 5'-flanking sequence. *Diabetes* 45:1563–1571.
313. Lochhead PA, Coghlan M, Rice SQJ, Sutherland C. 2001. Inhibition of GSK-3 selectively reduces glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression. *Diabetes* 50:937–946.
314. Metzger S, Begleibter N, Barash V, Drize O, Peretz T, Shiloni E, Chajek-Shaul T. 1997. Tumor necrosis factor inhibits the transcriptional rate of glucose-6-phosphatase in vivo

- and *in vitro*. *Metabolism* 46:579–583.
- 315. Metzger S, Goldschmidt N, Barash V, Peretz T, Drize O, Shilyansky J, Shiloni E, Chajek-Shaul T. 1997. Interleukin-6 secretion in mice is associated with reduced glucose-6-phosphatase and liver glycogen levels. *Am J Physiol Metab* 273:E262–E267.
 - 316. Ma R, Zhang W, Tang K, Zhang H, Zhang Y, Li D, Li Y, Xu P, Luo S, Cai W, Ji T, Katirai F, Ye D, Huang B. 2013. Switch of glycolysis to gluconeogenesis by dexamethasone for treatment of hepatocarcinoma. *Nat Commun* 4.
 - 317. Liu H-Y, Hong T, Wen G-B, Han J, Zuo D, Liu Z, Cao W. 2009. Increased basal level of Akt-dependent insulin signaling may be responsible for the development of insulin resistance. *Am J Physiol Metab* 297:E898–E906.

ADDENDUM A: ADDITIONAL DATA

A1. BWTG3 and HepG2 cells used in this study were tested for mycoplasma every quarter and were mycoplasma negative.

Routine testing for mycoplasma was done as this infection is difficult to detect as these microorganisms do not adhere to cell surfaces, do not contain a cell wall and cannot be seen with the naked eye as they do not cause visible turbidity. These micro-organisms are also able to avert the antibiotics commonly used for tissue culture in this study. Therefore, this kind of infection can go undetected for months without notice. Contamination of this nature affects cell growth as well as experimental results, thereby skewing the data, resulting in inaccurate reporting. For this reason, the cells in this study were tested once every 3 months. The Hoechst-dye stains only DNA containing nuclei. Therefore, any fluorescence found around the cells (would be visible as little spots between cells) would indicate contamination with mycoplasma.

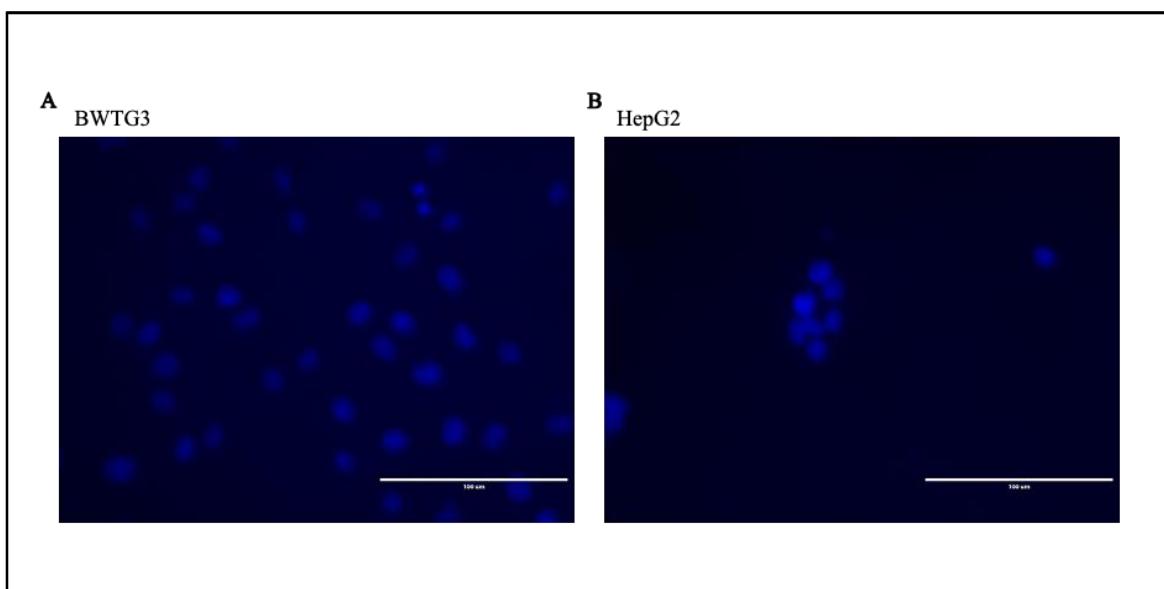


Figure A1. Mycoplasma negative BWTG3 (A) and HepG2 (B) cells. Cells were fixed with methanol and glacial acetic acid in a 3:1 ratio before staining with the DNA Hoechst 33258 dye (Sigma-Aldrich, SA). The Hoechst-dye only stains DNA-containing nuclei. Fluorescent images were obtained using the Olympus XI81 inverted fluorescent microscope.

A2. RNA isolated from BWTG3 and HepG2 cells were intact.

RNA concentration and purity were determined using the Nanodrop ND-1000. Further validation of the integrity of the RNA was done by separating RNA samples on a 1% (w/v) agarose gel stained with Nancy-520 (Sigma-Aldrich). The presence of undamaged 28S and 18S ribosomal bands indicates that the RNA can be used to continue with synthesis of cDNA.

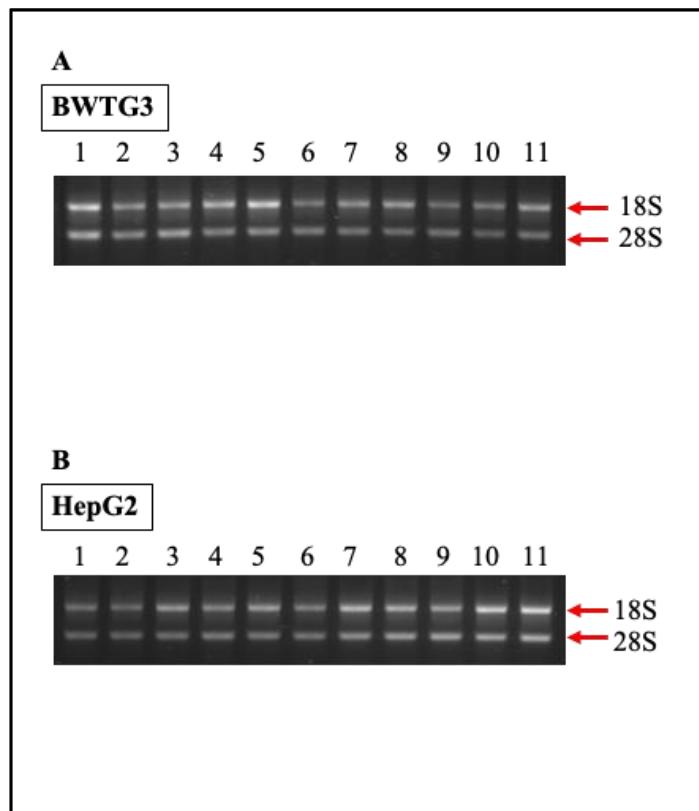


Figure A2. Representative 1% denaturing agarose gel indicating intact RNA isolated from BWTG3 and HepG2 cells. Total RNA was isolated from BWTG3 (A) and HepG2 (B) cells. RNA was isolated using Tri-reagent as described in Chapter 2, section 2.3. A total of 1 µg RNA was loaded onto the agarose gel and visualized with Nancy-520 nucleic acid stain. Lanes: 1) TNF- α ; 2) IL-6; 3) 10 nM dex; 4) 100 nM dex; 5) 1 µM dex; 6) 10 nM dex + TNF- α ; 7) 100 nM dex + TNF- α ; 8) 1 µM dex + TNF- α ; 9) 10 nM dex + IL-6; 10) 100 nM dex + IL-6; 11) 1 µM dex + IL-6

A3. The correct human G6Pase qPCR product was confirmed with agarose gel electrophoresis.

In order to determine that the correct product was amplified during qPCR, the product was separated using 3% (w/v) agarose gel electrophoresis and visualised with Nancy-520 nucleic acid stain. The size of the product was compared to a 100 bp DNA ladder as the amplicon was expected to be 141 bp in size. The melting curve for the qPCR product is also included below (Fig A3.2). Peaks found at 82 degrees represent the template containing samples and peaks seen between 72.00 and 74.00 degrees represent non-template controls. A possible reason for seeing the peaks at the lower temperature is primer dimer formation. The fact that these peaks are not present in the samples containing template suggests the primer is specific enough to use for amplification. Primer efficiency was also determined using a dilution series containing cDNA from untreated HepG2 cells and the standard curve can be seen in figure A3.3.

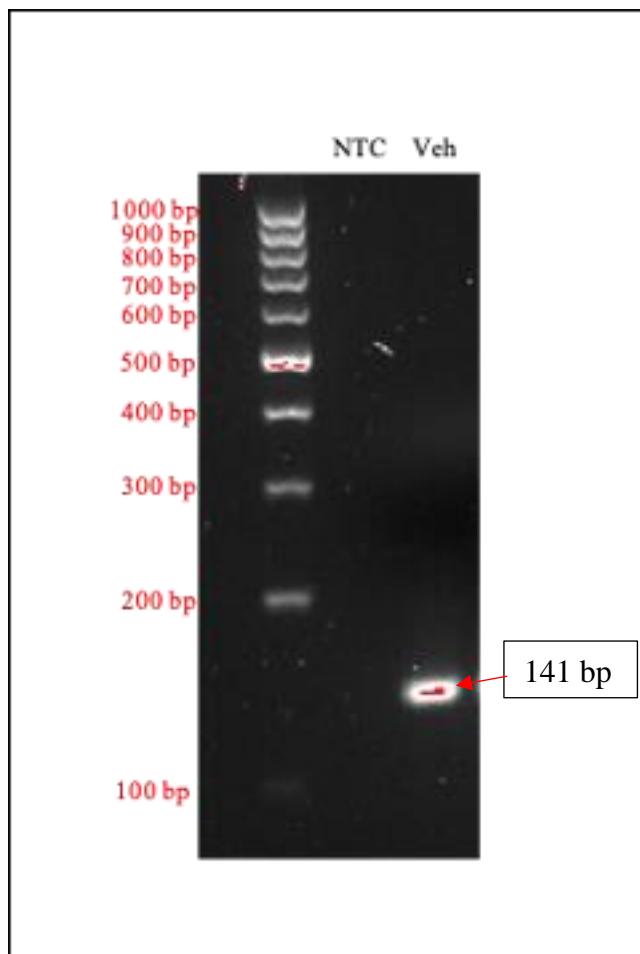


Figure A3.1. Representative agarose gel indicating qPCR product of human G6Pase gene used in this study. PCR products were subjected to agarose (3% w/v) gel electrophoresis and visualised with Nancy-520 nucleic acid stain. O'GeneRuler 100bp DNA ladder was used (ThermoFisher Scientific, USA); NTC: non-template control; Veh: sample treated with vehicle (DMEM-full).

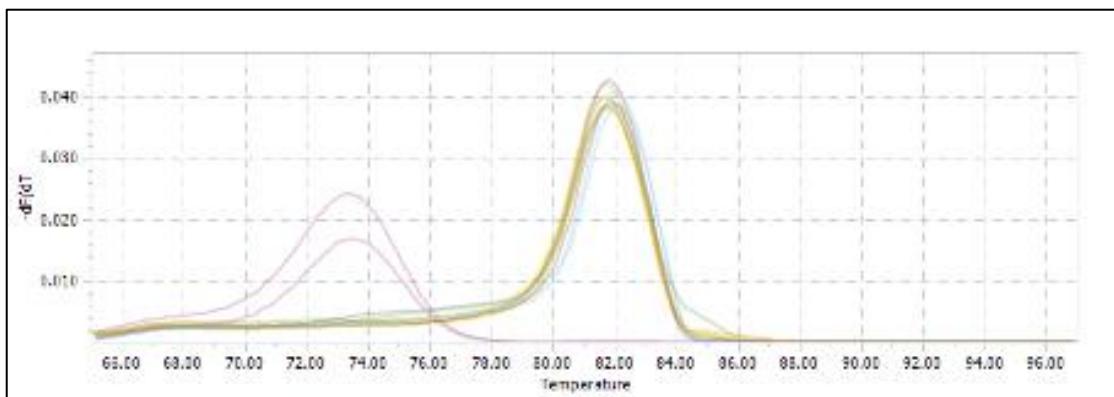


Figure A3.2. Melting curve for the above product gel. Peaks seen around 82.00 degrees indicate the samples containing template. The two peaks seen between 72.00 and 73.00 degrees represent the non-template control.

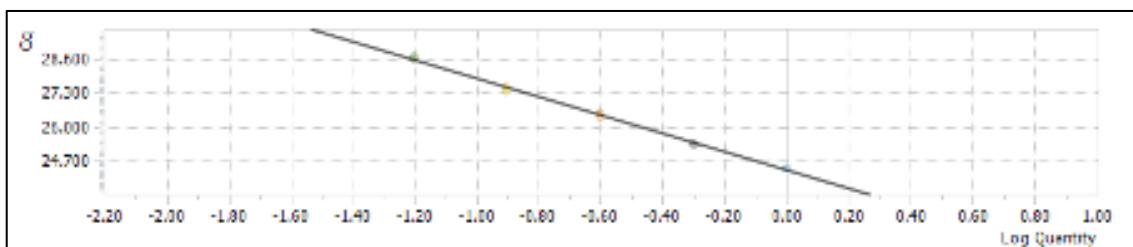


Figure A3.3. Standard curve used to determine primer efficiency of human G6Pase primers.