University of Alberta

REGULATION OF FOXO TRANSCRIPTION FACTORS BY OXIDATIVE STRESS: ROLE OF GLUTATHIONE

by

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DEDICATION

This work is dedicated to my most beloved person in this life

and my ultimate motivator towards success

My mother

Amnah Abdullah Jan Tkhta

ABSTRACT

FoxO transcription factors are downstream mediators of insulin effects that can bind to DNA and regulate transcription of multiple target genes. These target genes are known for their importance in several biological functions including energy homeostasis, longevity, tumour suppression and development. FoxO activity has been reported to be affected by insulin signaling and oxidative stress. Glutathione (GSH), a potent antioxidant and regulator of cellular redox state could therefore play a role in regulating FoxO activity. Herein, modulators of GSH levels were used to investigate the effect of GSH depletion on insulin-induced FoxO1/3a phosphorylation. HepG2 human hepatoma cells were exposed to diethyl maleate (DEM) to deplete thiols including glutathione, or buthionine sulfoximine (BSO) to inhibit the synthesis of glutathione, followed by insulin treatment, western blotting and immunodetection of FoxO1/3a phosphorylation and other insulin signaling targets. Our results show that DEM and BSO significantly attenuate insulin-induced FoxO1/3a phosphorylation. In addition, the mechanism of DEM attenuating phosphorylation of FoxO1/3a has been investigated. DEM was found to be associated with a general impairment of insulin signaling and an activation of stress-induced Jun N-terminal kinases, JNK, which had previously been shown to modulate FoxO activity. In conclusion, depletion of thiols, including glutathione modulates FoxO signaling by interfering with FoxO1/3a phosphorylation.

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LIST OF ABBREVIATIONS

5-SSA	5-Sulfosalicylic acid
AMPK	Adenosine monophosphate-activated protein kinase
APS	Ammonium peroxodisulfate
Bim	Bcl-2-like protein 11
BCA	Bicinchoninic acid
BSO	Buthionine sulfoximine
JNK	c-Jun N-terminal kinase
CHIP	C-terminus of Hsc70-interacting protein
CK1	Casein kinase 1
COP1	Constitutive photomorphogenesis protein 1
CBP/P300	CREB-binding protein/P300
CDK2	Cyclin-dependent kinase 2
DEM	Diethyl maleate
DMSO	Dimethyl sulfoxide
DTNB	Dithionitrobenzoic acid
DYRK1	Dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1
DMEM	Dulbecco's modified Eagle's medium
FOX	Forkhead box
G6Pase	Glucose-6-phosphatase
GSSG	Glutathione disulfide
GSH	Glutathione
GSHe	Glutathione ethyl ester
IKKβ	IκB kinase beta
IRS	Insulin receptor substrate

MST	Mammalian Ste20-like kinase
MDM2	Mouse double minute 2 homolog
TEMED	N,N,N',N'-Tetramethylethylenediamine
NES	Nuclear export sequence
NLS	Nuclear localization signal
PCAF	P300/CBP-associated factor
PUMA	p53 upregulated modulator of apoptosis
PGC-1α	Peroxisome proliferator-activated receptor-gamma co-activator 1
PTEN	Phosphatase and tensin homolog
PBS	Phosphate-buffered saline
PIP3	Phosphatidylinositol (3’,4’,5’)-trisphosphate
PI3K	Phosphoinositide 3’-kinase
PEPCK	Phosphoenolpyruvate carboxykinase
PDPK	Phosphoinositide-dependent protein kinase
PH domain	Pleckstrin homology domain
PRMT1	Protein arginine methyltransferase 1
PKB	Protein kinase B (an alternate name for Akt)
PP2A	Protein phosphatase 2A
ROS	Reactive oxygen species
SKP2	S-phase kinase-associated protein 2
SGK	Serum and glucocorticoid-induced kinase
Sirt1	Sirtuin 1
MnSOD	Manganese superoxide dismutase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNB	Thionitrobenzonate

TBS	Tris-buffered saline
TBST	Tris-buffered saline plus tween
USP7/HAUSP	Ubiqutin-specific-processing protease 7 / herpesvirus-associated ubiquitin-specific protease

CHAPTER 1

INTRODUCTION

1.1 Background: Oxidative Stress and Insulin Signaling

Oxidative stress is a common physiological state that has been linked with several life threatening conditions including diabetes, cancer, cardiovascular, lung and neurological diseases (Markesbery 1997; Baynes and Thorpe 1999; Dhalla et al. 2000; MacNee 2001; Reuter et al. 2010). Oxidative stress occurs as a consequence of imbalance between oxidants and antioxidants towards the former (Sies. 1985). Oxygen consumption is essential for human life, yet upon respiration some oxygen is converted to reactive oxygen species (ROS) which are harmful substances that can damage DNA, proteins and lipids. These ROS are mainly generated from mitochondria upon utilization of oxygen to produce energy through the electron transport chain, then released into the cytoplasm. If the generated ROS are not neutralized by antioxidants, oxidative stress will ensue and cell signaling processes will be affected (Thannickal and Fanburg 2000). Depending on the level of ROS present inside cells, the redox status would be altered. This alteration in cellular redox status can influence a multitude of upstream and downstream signaling cascades (Kamata and Hirata 1999). Of interest, a series of upstream serine/threonine kinases are activated under oxidative stress environments (Griendling et al. 2000). As a consequence, a series of downstream transcription factors would also be affected.

The insulin signaling pathway is an essential physiological regulator of cell survival and metabolism. Upon insulin binding to the insulin receptor, auto-phosphorylation of tyrosine residues in the beta subunits occurs resulting in recruitment and phosphorylation of several insulin receptor substrates (IRS). This leads to activation of a variety of signaling cascades including the PI3K (Phosphoinositide 3’-kinase) pathway, Akt/PKB (Protein kinase B), and

MAPK (Mitogen-activated protein kinases)/ERK (Extracellular signal-regulated kinases). Akt activation causes phosphorylation of several downstream proteins that mediate insulin effects (Song et al. 2005). Of interest to this work, one group of these proteins is a family of transcription factors known as Fox (Forkhead box) class O proteins that are heavily influenced by insulin signaling.

1.2 FoxO Transcription Factors

1.2.1 Overview

FoxOs are a family of DNA-binding proteins that regulate transcription of specific target genes. These target genes are known for their importance in stress resistance, glucose and lipid metabolism, survival, development and tumor suppression (Calnan and Brunet 2008; Becker et al. 2010; Monsalve and Olmos 2011). A list of FoxO target genes is provided in Figure 1.1. The DNA binding sequence motif recognized by FoxO proteins and present in the promotor regions of these target genes has been identified to be 5'-(G/C)(A/T)AAA(C/A)A-3' (Furuyama et al. 2000). The FoxO family consists of four members highly conserved across species, FoxO1, FoxO3a, FoxO4 and FoxO6. FoxO proteins mainly act as transcriptional activators, however they can also act as transcriptional repressors (Ramaswamy et al. 2002). Of importance to our research, this family of proteins are heavily regulated by insulin signaling and oxidative stress as shown in the following sections.

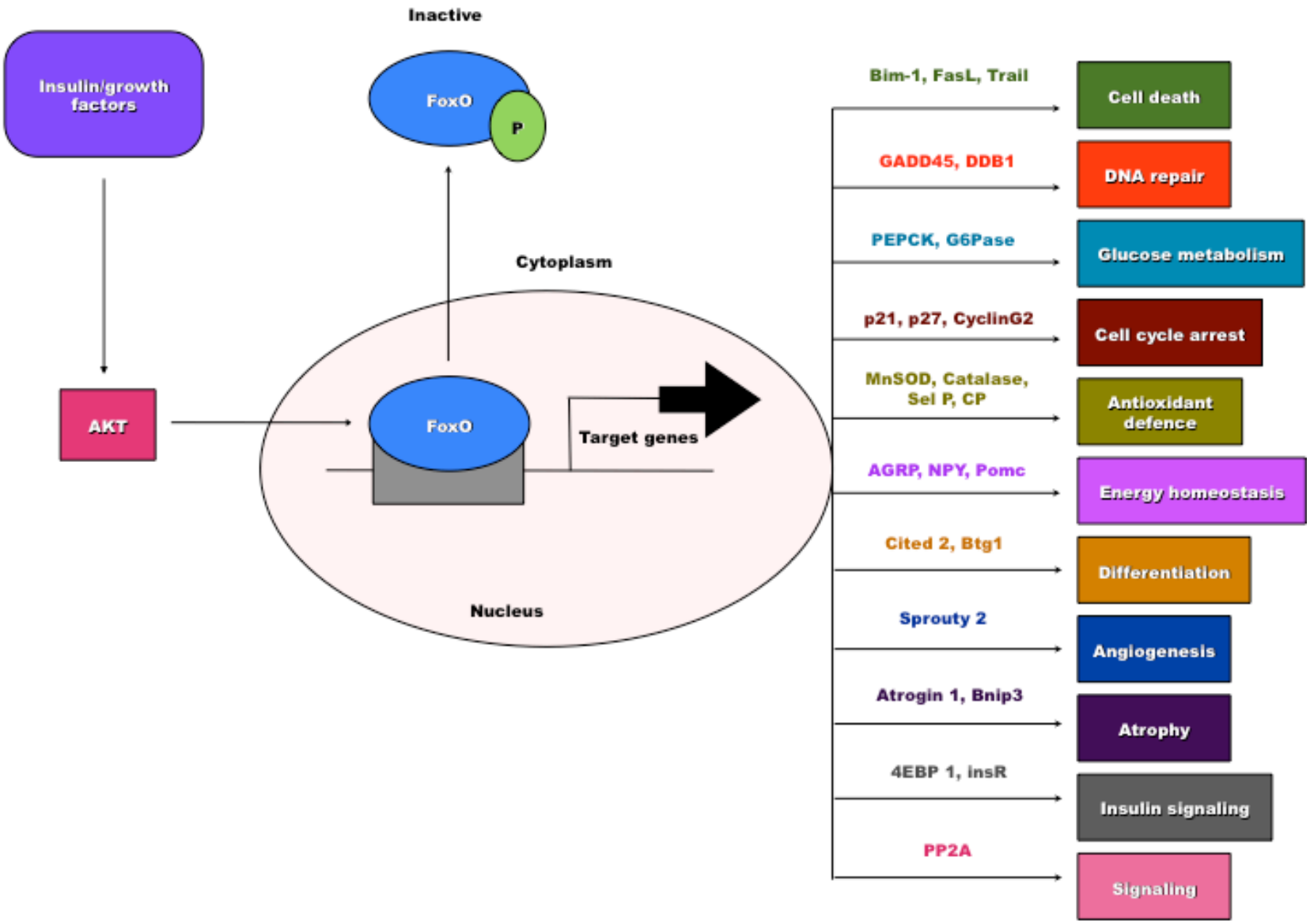
Inactive
Insulin/growth factors
FoxO
P
Cytoplasm
AKT
FoxO
Target genes
Nucleus
Bim-1, FasL, Trail
Cell death
GADD45, DDB1
DNA repair
PEPCK, G6Pase
Glucose metabolism
p21, p27, CyclinG2
Cell cycle arrest
MnSOD, Catalase, Sel P, CP
Antioxidant defence
AGRP, NPY, Pomc
Energy homeostasis
Cited 2, Btg1
Differentiation
Sprouty 2
Angiogenesis
Atrogin 1, Bnip3
Atrophy
4EBP 1, insR
Insulin signaling
PP2A
Signaling

Figure 1.1 FoxO target genes. Insulin or growth factors activate Akt which in turn phosphorylate FoxO proteins, resulting in FoxO cytoplasmic localization (inactivation). Active FoxO proteins inside the nucleus recognize and bind to a DNA sequence (5'-(G/C)(A/T)AAA(C/A)A-3') present in several target genes listed above, up-regulating their transcription except Pomc and PP2A (down-regulation); and Sprouty 2 (unknown effect). FasL, Fas ligand (Brunet et al. 1999); Trail, TNF-related apoptosis-inducing ligand (Modur et al. 2002); GADD45, growth arrest and DNA damage-inducible protein 45 (Tran et al. 2002); DDB1, damage-specific DNA-binding protein 1 (Ramaswamy et al. 2002; Chen et al. 2008); PEPCK, phosphoenolpyruvate carboxykinase (Nakae et al 2002); G6Pase, glucose-6-phosphatase (Puigserver et al. 2003); MnSOD, manganese superoxide dismutase (Kops et al. 2002); Sel P, selenoprotein P (Walter et al. 2008); CP, ceruloplasmin (Leyendecker et al. 2011); AGRP, agouti-related protein (Kitamura et al. 2006); NPY, neuropeptide Y (Kim et al. 2006); Pomc, pro-opiomelanocortin (Kitamura et al. 2006); Cited2, Cbp/p300-interacting transactivator 2 (Bakker et al. 2007); Btg, B-cell translocation gene 1 (Bakker et al. 2004); Bnip3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Mammucari et al. 2007); 4EBP, 4E-binding protein 1 (Junger et al. 2003); InsR, insulin receptor (Puig and Tjian 2005); PP2A, protein phosphatase 2A (Ni et al. 2007).

1.2.2 FoxO expression profile

FoxO1, FoxO3 and FoxO4 isoforms are widely expressed in most tissues. However, in some tissues, specific FoxO isoforms are more predominant than the others. For instance, FoxO1 is highly expressed in adipose tissue. FoxO3a is highly expressed in heart and brain. FoxO4 is abundant in skeletal and cardiac muscle, while FoxO6 is mainly expressed in the brain (Greer and Brunet 2005; Monsalve and Olmos 2011). In addition, recent data have shown that both FoxO1 and FoxO3a are highly expressed in liver (Salih and Brunet 2008; Valenti et al. 2008).

1.2.3 Regulation by Oxidative Stress and Post-translational Modifications

FoxO transcription factors are regulated by various external stimuli, ranging from insulin and insulin-like growth factors (IGF), to growth hormones and stressful stimuli, such as oxidants (van der Horst and Burgering 2007). The regulatory effect of these stimuli can either lead to activation or inactivation of FoxO proteins, based on the changes in FoxO subcellular localization these stimuli elicit. For instance, insulin signaling results in FoxO phosphorylation and subsequent nuclear exclusion which renders FoxO proteins inactive (Arden and Biggs 2002). On the other hand, oxidative stress in general induces FoxO translocation from the cytoplasm to the nucleus (Brunet et al. 2004). Depending on the level of oxidative stress, FoxO proteins either up-regulate pro-survival (stress-resistance-mediating) genes or up-regulate pro-apoptotic genes, leading to cell death (Calnan and Brunet 2008). The following sections will contain detailed information about FoxO post-translational modifications including phosphorylation, acetylation, ubiquitination, methylation and glycosylation.

1.2.3.1 FoxO phosphorylation

Phosphorylation is the most common and best-understood post-translational modification that is responsible for FoxO regulation and subcellular localization. There are several sites on FoxO proteins that are subjected to phosphorylation by serine/threonine kinases. Upon phosphorylation by a variety of kinases below, FoxO is subjected to 14-3-3 binding to facilitate CRM1-mediated nuclear export, cytoplasmic localization and inactivation (Huang and Tindall 2007). Akt and serum glucocorticoid-induced kinase (SGK), casein kinase 1 (CK1), dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase (DYRK1), cyclin-dependent kinase 2 (CDK2), and IκB kinase (IKKβ) are all negative regulators of FoxO activity, promoting FoxO cytoplasmic localization. Of interest to this work, insulin signaling activates Akt, which phosphorylates FoxO1 at threonine24 and FoxO3a at threonine32, resulting in cytoplasmic localization and inactivation of these proteins (Tzivion et al. 2011). On the other hand, adenosine monophosphate-activated protein kinase (AMPK) phosphorylates FoxO3, leading to increased transcriptional activity independent of FoxO localization, however the exact mechanism is unclear (Greer et al. 2007). Furthermore, oxidative-stress-dependent mammalian sterile 20-like kinase 1 (MST1) was reported to bind and phosphorylate FoxO, leading to nuclear accumulation and activation (Lehtinen et al. 2006). Similarly, c-Jun kinase (JNK) also phosphorylates FoxO4 leading to nuclear accumulation and enhanced FoxO4 activity (Essers et al. 2004). JNK has also been shown to phosphorylate 14-3-3, interrupting the binding of FoxO3 with 14-3-3 and thereby blocking Akt signaling effects (Sunayama et al. 2005). In addition, oxidative stress, a known activator of the JNK pathway, was reported to decrease insulin-induced Akt activation after long incubation with hydrogen peroxide in HIT cells, which leads to a decrease in Akt-dependent

phosphorylation of FoxO1, indicating a possible indirect role for JNK in affecting FoxO phosphorylation (Kawamori et al. 2006). However, the exact mechanism is not known. Interestingly, oxidative stress has also been reported to induce PI3K pathway and Akt activity (Barthel and Klotz 2005). Finally, as a reverse mechanism to FoxO phosphorylation, cellular serine/threonine protein phosphatase 2A (PP2A) was reported to be involved in de-phosphorylating FoxO1 (Yan et al. 2008).

1.2.3.2 FoxO acetylation

FoxO acetylation/deacetylation is an important post-translational modification also affecting subcellular localization. FoxO proteins are acetylated on lysine residues in response to oxidative stress. Several acetylases and deacetylases are known to regulate FoxO activity. Nuclear CREB-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF) are transcriptional co-activators with histone acetyltransferase (HAT) activity. They are able to acetylate lysine residues within FoxO proteins. Wild-type CBP expression has been shown to increase FoxO1 transcriptional activity (Daitoku et al. 2004). In contrast, FoxO4 acetylation by CBP in the presence of stressful stimuli was shown to attenuate FoxO4 transcriptional activity (van der Horst and Burgering 2007) possibly due to impaired DNA-binding ability (Matsuzaki et al. 2005). FoxO acetylation is augmented under oxidative stress conditions (Brunet et al. 2004). Acetylated FoxO proteins appear to reside in the nucleus (Kitamura et al. 2005). On the other hand, FoxO deacetylation is mainly carried out by sirtuins (Sirt1) and class III histone deacetylases (HDACs) (Zhao et al. 2011). Sirt1 was reported to increase FoxO3-related cell cycle arrest and stress resistance effects yet interestingly decrease FoxO3-related cell death effect

(Brunet et al. 2004). Resveratrol, a natural polyphenol, was reported to increase FoxO1 nuclear retention by inhibiting Sirt1 activity, resulting in increased expression of FoxO target genes (Qiang et al. 2010). In general, the consequence of FoxO acetylation on FoxO activity appears to be controversial and not well understood. This may be due to the high sensitivity of FoxO to the overall extent of oxidative stress-induced acetylation (Calnan and Brunet 2008).

1.2.3.3 FoxO ubiquitination

Ubiquitination is a common post-translational modification that involves the attachment of ubiquitin to lysine residues of corresponding proteins. FoxO transcription factors undergo two levels of ubiquitination, mono and polyubiquitination. As mentioned in section 1.2.3.1, insulin signaling and Akt activation leads to FoxO phosphorylation, nuclear export and cytoplasmic localization. In addition, this Akt-dependent phosphorylation has been shown to promote subsequent degradation of FoxO proteins by the proteasomal system following their ubiquitination (Matsuzaki et al. 2003; Plas and Thompson 2003). Furthermore, ERK and IKKβ were also reported to induce FoxO3a proteolysis (Hu et al. 2004; Yang et al. 2008). Several ubiquitin E3 ligases are reported to ubiquitinate FoxO proteins. Polyubiquitination is carried out by S-phase kinase-associated protein 2 (Skp2). In FoxO1, this process has been found to be dependent on pre-phosphorylation at serine 256 by Akt (Huang et al. 2005). Mouse double minute 2 homolog (MDM2) has been shown to polyubiquitinate FoxO1 and FoxO3a (Yang et al. 2008; Fu et al. 2009). MDM2 was also reported to monoubiquitinate FoxO4, resulting in nuclear localization and increased transcriptional activity under oxidative stress conditions (Brenkman et al. 2008). Herpes-virus-associated ubiquitin-specific protease (HAUSP) on the other hand is a

FoxO deubiquitinating enzyme that binds to FoxO4 and releases this protein from monoubiquitnation in response to oxidative stress (Brenkman et al. 2008). Another recently identified ubiquitin-proteasome pathway that affects FoxO1 was discovered to be mediated by constitutive photomorphogenesis protein 1 (COP1) (Kato et al. 2008). Lastly, the C-terminus of Hsc70-interacting protein (CHIP) has been demonstrated to promote FoxO1 degradation in smooth muscle cells (Li et al. 2009).

1.2.3.4 FoxO Methylation and glycosylation

Other recently discovered FoxO post-translational modifications include methylation and glycosylation. Methylation of FoxO1 by protein arginine methyltransferase 1 (PRMT1) has been reported to block Akt-mediated phosphorylation of FoxO1 at serine253 in mouse, which is corresponding to serine256 in humans (Yamagata et al. 2008). PRMT1 knockdown was associated with FoxO1 cytoplasmic localization and polyubiquitination, while methylated FoxO1 was present in the nucleus (Yamagata et al. 2008). In addition, FoxO3 methylation by Set9 methyltransferase at lysine270 resulted in inhibition of DNA-binding activity and transactivation of FoxO3 (Xie et al. 2012). Lastly, FoxO1 has been shown to be a target of O-glycosylation. This modification of FoxO1 increases the gene expression of Glucose-6-phosphatase (G6pase). However, it does not affect FoxO1 subcellular distribution (Kuo et al. 2008). A summary of post-translational modifications and their effects on FoxO transcription factors is provided in Table 1.1.

Table 1.1 Summary of FoxO post-translational modifications based on stimulus (see text for refs)

Stimulus	Enzyme(s)	Modification	Effect on FoxO transcriptional activity
Insulin signaling/ growth factors	Akt	Phosphorylation	-
	SGK	Phosphorylation	-
	CK1	Phosphorylation	-
	CDK2	Phosphorylation	+
	ERK	Phosphorylation	-
	IKKβ	Phosphorylation	-
	AMPK	Phosphorylation	+
	Skp2	Poly-ubiquitination	-
	MDM2	Poly-ubiquitination	-
Oxidative stress	MST1	Phosphorylation	+
	JNK	Phosphorylation	+
	CBP/P300	Acetylation	-
	Sirt1	De-acetylation	+
	USP7/HAUSP	Mono-deubiquitnation	-
	MDM2	Mono-ubiquitination	+
Undetermined	COP1, CHIP	Poly-ubiquitination	-
	PRMT1	Methylation	+
	Set9	Methylation	-
	Unknown	Glycosylation	+
	DYRK1	Phosphorylation	-

Abbreviations: SGK, serum and glucocorticoid-induced kinase; CK1, casein kinase 1; CDK2, cyclin-dependent kinase 2, ERK, extracellular-signal-regulated kinases; IKKβ, IκB kinase; AMPK, adenosine monophosphate-activated protein kinase; Skp2, S-phase kinase-associated protein 2; MDM2, mouse double minute 2 homolog; MST1, mammalian ste20-like kinase 1; JNK, c-Jun N-terminal kinase; CBP/P300, CREB-binding protein/P300; Sirt1, sirtuin 1; USP7/ HAUSP, ubiqutin-specific-processing protease 7 / herpesvirus-associated ubiquitin-specific protease; COP1, constitutive photomorphogenesis protein 1; CHIP, C-terminus of Hsc70-interacting protein; PRMT1, protein arginine methyltransferase 1; DYRK1, dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1.

1.2.4 FoxO regulation and interaction with binding partners

In addition to regulation by stress and insulin, FoxO transcription factors are regulated by interacting with binding partners. These interacting partners are mainly co-activators of FoxO-dependent gene expression. For instance, FoxO3 has been reported to interact with p53, a known tumor suppressor in humans (Brunet et al. 2004). This co-activator has been recently found to transactivate the FoxO3 gene (Renault et al. 2011). Another FoxO-interacting partner important in cancer is beta-catenin, a known proto-oncogene in the Wnt signaling pathway. Under oxidative stress conditions, β-catenin interacts with FoxO leading to increased transactivation activity of FoxO (Essers et al. 2005). Furthermore, in the presence of transforming growth factor beta, SMAD transcription factors interact with FoxO forming a complex that up-regulates p21 leading to G1 arrest (Seoane et al. 2004). This interaction is reversed by insulin signaling. CCAAT/enhancer-binding protein alpha and beta both interact and act as co-activators of FoxO (Christian et al. 2002; Qiao and Shao 2006). A FoxO1 co-repressor named FCoR was reported to repress FoxO1 activity by enhancing FoxO1 acetylation (Nakae et al. 2012). FoxO4 interacts with myocardin resulting in inhibition of myocardin activity (Liu et al. 2005). In addition, FoxO interferes with Notch signaling pathway through direct interaction with Csl, leading to increased activity of Notch target genes (Kitamura et al. 2007). Hoxa5 and Hoxa10 interact with FoxO resulting in increased insulin-like growth factor binding protein 1 (IGFBP1) promoter activity (Foucher et al. 2002; Kim et al. 2003). FoxO1a was also reported to act as a co-activator of signal transducer and activator of transcription 3 (STAT 3) (Kortylewski et al. 2003). Small heterodimer partner (SHP) interacts with FoxO1 resulting in repression of G6Pase transcription (Yamagata et al. 2004).

Other important FoxO interacting partners influencing gluconeogenesis include peroxisome proliferator-activated receptor-gamma co-activator 1 (PGC-1α) and peroxisome proliferator-activated receptor gamma (PPAR-γ). PGC-1α interaction with FoxO stimulates gluconeogensis (Puigserver et al. 2003), while insulin signaling impairs this interaction (Li et al. 2007). On the other hand, FoxO1 interaction with PPAR-γ is antagonistic. FoxO1 trans-represses PPAR-γ inside the nucleus by direct interaction with PPAR-γ response elements (Fan et al. 2009). Hepatocyte nuclear factor 4 (HNF4) has been reported to activate FoxO1-dependent G6Pase and selenoprotein P transcription (Hirota et al. 2008; Speckmann et al. 2008).

Several hormone receptors and nuclear receptors have been reported to interact with FoxO transcription factors. The androgen receptor interacts with FoxO proteins, repressing their action in an Akt-independent manner (Li et al. 2003). FoxO binds to estrogen response elements, resulting in increased transactivation activity of estrogen receptor alpha and repression of FoxO-mediated transactivation activity (Schuur et al. 2001). Progesterone receptor A and progesterone receptor B interact with FoxO. However, their effect on transactivation activity of FoxO appears to be cell type-dependent (Kim et al. 2005). FoxO interacts with thyroid hormone receptor stimulating its transactivation activity (Zhao et al. 2001). Follicle stimulating hormone receptor (FSHR) also interacts with FoxO1a, yet no specified effect was reported (Nechamen et al. 2007). Transcriptional activity of nuclear receptors such as constitutive androstane receptor (CAR) and preganane x receptor (PXR) is co-activated by FoxO1, while FoxO1-mediated transcription is co-repressed by CAR and PXR (Kodama et al. 2004). Also, retinoic acid receptor (RAR) activity

is stimulated by FoxO (Zhao et al. 2001). A summary of FoxO interacting partners is provided in Table 1.2.

Table 1.2 Summary of FoxO interacting partners (see text for refs)

FoxO interacting partner	Effect
AR	Decreased FoxO activity
CAR	Increased CAR activity AND decreased FoxO activity
PXR	Increased PXR activity AND decreased FoxO activity
CEBP-α/β	Increased FoxO activity
ER	Decreased FoxO activity
FCoR	Decreased FoxO activity
FoxG1	Decreased FoxO activity
FSHR	Unspecified
HNF4	Increased FoxO1-dependent G6Pase and selenoprotein P transcription
Hoxa5/Hoxa10	Increased IGFBP1 promoter activity
Myocardin	Decreased myocardin activity
Notch/Csl	Increased Notch target gene expression
p53	Increased FoxO activity
PGC-1α	Increased FoxO activity
PPAR-γ	Increased FoxO activity
PR	Cell type-dependent
RAR	Increased RAR activity
SHP	Decreased FoxO-mediated G6Pase transcription
SMAD	Synergistic upregulation of p21; cell cycle arrest
STAT3	Increased STAT3 activity
TR	Increased TR activity
β-catenin	Increased FoxO activity

Abbreviations: AR, androgen receptor; CAR/PXR, constitutive androstane receptor / preganane x receptor; CEBP-α/β. enhancer-binding protein alpha/beta; ER, estrogen receptor; FCoR, FoxO1-CoRepressor; FoxG1, forkhead box protein G1; FSHR, follicle stimulating hormone receptor; HNF4, hepatocyte nuclear factor 4; Hoxa5/10, homeobox proteins 5/10; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR-γ, peroxisome proliferator-activated receptor-gamma; PR, progesterone receptor; TR, thyroid receptor; RAR, retinoic acid receptor; SHP, small heterodimer partner; STAT3, signal transducer and activator of transcription 3; TR, thyroid receptor.

1.2.5 Physiological roles of FoxO

FoxO transcription factors are involved in the regulation of several target genes linked with pathological processes, and in the control of a variety of cell signaling events essential for survival.

1.2.5.1 Diabetes and inflammation

Hepatic overproduction of glucose is a factor contributing to hyperglycemia in diabetes. Proper insulin signaling protects against hyperglycemia by inhibiting the formation of two important gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Pilkis and Granner 1992). The expression of these two enzymes is known to be up-regulated by FoxO proteins (Barthel et al. 2005). Indeed, FoxO1 knockout mice showed lower expression levels of PEPCK and G6Pase (Altomonte et al. 2003). Therefore, FoxO transcription factors have been categorized as downstream mediators of insulin effects (Barthel et al. 2005). This is also due to FoxO sensitivity towards insulin-induced PI3K-Akt activation and oxidative stress. As mentioned above, Akt activation leads to FoxO phosphorylation and cytoplasmic localization (inactivation), while oxidative stress may lead to increased FoxO activity and nuclear localization. However, since the response of FoxO to oxidative stress is variable based on the level and duration of stress, other findings showed that stress-induced PI3K signaling resulting in decreased FoxO activity (Bartholome et al. 2010). Increasing evidence implies that oxidative stress is directly linked with diabetes (Maritim et al. 2003). High activity of FoxO1 due to oxidative stress leads to increased expression of pro-inflammatory cytokine IL-1β (Su et al. 2009) and TNF-α (Behl et al. 2009). In addition, FoxO1 stimulates toll-like

receptor 4 (TLR4) signaling which is associated with inflammation (Fan et al. 2010). Also, high levels of TNF-α were associated with FoxO1 nuclear localization and aggravated cartilage resorption (Alblowi et al. 2009). These findings have correlated FoxO directly to insulin-resistance and type 2 diabetes, and encouraged the development of FoxO inhibitors for type 2 diabetes (Nagashima et al. 2010).

1.2.5.2 Cancer and apoptosis

FoxO proteins have been suggested as potential targets for future cancer therapy (Maiese et al. 2008). The initial hint suggesting a role of FoxO in cancer was discovered after identifying three FoxO mutated genes highly expressed in tumors: FoxO1 previously known as FKHR in rhabdomyosarcomas, FoxO3 previously known as FKHRL1 and FoxO4 previously known as AFX in acute myeloid leukemias (Borkhardt et al. 1997; Hillion et al. 1997). FoxO1 was reported to be inactive in PTEN-negative carcinomas (Nakamura et al. 2000). And upon constitutive expression of FoxO1 in PTEN-null cells, tumorigenesis is hindered (Ramaswamy et al. 2002). In addition, expression of the oncogenic enzyme IkappaB Kinase (IKK) inhibited FoxO3 activity in breast cancer (Hu et al. 2004). Similarly, constitutively active FoxO4 inhibited HER2-activated tumors (Yang et al. 2005). All these findings indicate that FoxO transcription factors play a key role in cancer as tumor suppressors. Additional levels of FoxO anti-cancer properties can be envisioned by FoxO interaction with tumor suppressors such as p53 (Brunet, Sweeney et al. 2004), SMAD (Seoane et al. 2004), and RUNX3 (Yamamura et al. 2006); and with oncogenes such as β-catenin which enhances FoxO ability to inhibit cell cycle progression (Essers et al. 2005). Another means of fighting cancer is up-regulation of pro-apoptotic markers.

FoxO3 has been reported to upregulate FasL and the corresponding signaling cascade (Brunet et al. 1999). Decreased activity of FoxO1 and FoxO3 in a prostate cancer cell line was associated with increased expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Modur et al. 2002). Also, FoxO proteins induce the expression of pro-apoptotic Bcl2-family member Bim (Dijkers et al. 2000). Another recently identified possible mediator of FoxO anti-cancer effect is Sprouty2, an antagonist of receptor tyrosine kinase signaling and ERK signaling (Bundschu et al. 2006). Sprouty2 knockdown was associated with similar cancer-related phenotypic consequences to FoxO deletions (Frank et al. 2009), highlighting Sprouty2 as a potential target of FoxO in endothelial cell (Paik et al. 2007). However, the exact mechanism of interaction between FoxO and Sprouty2 is unclear.

1.2.5.3 Muscle atrophy

Muscle atrophy can be considered as a symptom of several systemic diseases that are associated with rapid protein degradation via ubiquitination. Atrogin-1 has been identified as a Ub-protein ligase (E3) and a marker for atrophying muscles (Gomes et al. 2001). Interestingly, increased FoxO activity has been shown to induce the expression of atrogin-1 (Sandri et al. 2004). Heat shock protein 70 (Hsp70) has been reported to negatively regulate FoxO signaling in skeletal muscles by inhibiting FoxO3-induced promotor activation of atrogin-1 (Senf et al. 2010). These findings highlight FoxO as a potential target for debilitated muscles treatment or prevention.

1.2.5.4 Aging and development

A plethora of target genes regulated by FoxOs have nominated the latter as important regulators of cellular homeostasis, growth and survival. Expression of p27(KIP1), a known cell cycle inhibitor, has been reported to be downregulated upon insulin-induced FoxO phosphorylation, i.e. inactivation of FoxO (Dijkers et al. 2000; Medema et al. 2000). The growth arrest and DNA damage (Gadd45) gene has also been shown to be induced by FoxO, reflecting the ability FoxO factors to initiate stress resistance and DNA repair, which boosts organismal longevity (Tran et al. 2002). In addition, FoxOs can up-regulate the pro-apoptotic Bim protein through direct activation of the Bim promotor by two FoxO binding sites (Gilley et al. 2003). Furthermore, FoxO has been shown to induce manganese superoxide dismutase (MnSOD) expression during oxidative stress, which results in organismal protection from ROS (Kops et al. 2002). Silencing either FOXO1 or FOXO3a gene expression resulted in significant endothelial cell migration and angiogenesis. In parallel, constitutively active FoxO1 and FoxO3 down-regulated eNOS expression (Potente et al. 2005). These findings illustrate another level of cellular development control by FoxO.

1.3 Glutathione

1.3.1 Overview

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that plays several essential roles in living cells (Sies 1999). The highly nucleophilic sulfhydryl group (-SH) allows GSH to directly react with ROS neutralizing their harmful effects. In addition, GSH can act as a substrate of glutathione peroxidases to reduce peroxides, and as a substrate of glutathione-S-transferases to be coupled to reactive electrophilic compounds. In addition, GSH is present in millimolar

concentrations in most cells (Dickinson and Forman 2002). These findings have contributed to GSH being considered as a master antioxidant. Another important role of GSH is control and maintenance of cellular redox status and redox signaling (Filomeni et al. 2002). Through a complex thiol-exchange system, a balance between glutathione/glutathione disulfide (GSH/ GSSG) is able to maintain a proper redox environment for cells to proliferate in a healthy manner. Indeed, GSH depletion, leading to an imbalance of GSH/GSSG levels, has been associated with several diseases (Townsend et al. 2003). The following sections will serve to elucidate an overview of GSH synthesis, regeneration and protein-S-glutathiolation.

1.3.2 Synthesis and regeneration

GSH biosynthesis occurs in all living cells in two adenosine-5'-triphosphate (ATP)-dependent steps (Zhang and Forman 2012). The first step is the rate-limiting step which is catalyzed by the enzyme glutamate cysteine ligase (GCL), also known as γ-glutamylcysteine synthetase, and involves the attachment of L-cysteine to the side-chain (γ-) carboxyl group of L-glutamate. The second step is catalyzed by GSH synthetase: glycine is added to γ-glutamylcysteine. Under oxidative stress conditions, GSH is oxidized non-enzymatically or as a co-factor of GSH peroxidase, resulting in GSSG formation. This GSSG can be reduced back to two molecules of GSH in the presence of NADPH and glutathione reductase (GR) resulting in regeneration of GSH. In addition, glutaredoxins and thioredoxins are able to reduce protein thiols at the expense of GSH, thereby oxidizing GSH back to GSSG.

1.3.3 Role in upholding cellular redox state and in the cellular response to oxidative stress

Most cell lines are enriched with several redox systems that are independently regulated (Jones 2006). Commonly found redox systems are NADPH/$NADP^+$, NADH/NAD^+, glutaredoxin(red)/ glutaredoxin(ox), thioredoxin(red)/thioredoxin(ox) and most importantly the GSH/GSSG redox system. The GSH to GSSG ratio was established as a good indicator of cellular stress levels, with lower ratios indicative of higher stress and vice versa (Kemp et al. 2008). Redox systems generally work together to neutralize oxidative stress and maintain a healthy cellular redox state (Holmgren, Johansson et al. 2005). Under conditions of overwhelming oxidative stress, the cellular redox potential is altered, resulting in several changes in cell signaling events (Schafer and Buettner 2001). Specifically, the GSH/GSSG redox system has been reported to have a regulatory effect on the activities of several cellular proteins (Giustarini et al. 2004; Meyer 2008). This regulatory effect of GSH/GSSG on proteins is mainly achieved through protein-S-glutathiolation, particularly on reactive cysteinyl residues (Biswas et al. 2006) as discussed in the next section.

1.3.4 Glutathiolation

S-glutathiolation is a reversible reaction in which reactive cysteine residues in proteins are conjugated with glutathione. Although this reaction is known to occur under oxidative or nitrosative stress conditions (Klatt and Lamas 2000), it has been shown that protein-S-glutathiolation can occur also under normal physiological conditions, for example in response to growth factors stimulation (Wang et al. 2001), indicating the importance of S-glutathiolation in redox signaling and protein activity. Indeed, several proteins have been reported to have their activity inhibited upon S-glutathiolation (Cross and Templeton 2004; Pan and Berk 2007) or

potentiated (Adachi et al. 2004; Adachi et al. 2004). In addition, protein-S-glutathiolation has been linked with several human diseases (Giustarini et al. 2004; Newman et al. 2007; Dalle-Donne et al. 2008; Shelton and Mieyal 2008). Overall, protein-S-glutathiolation is an important new area of study that requires further research and investigation.

1.4 Rationale, Hypothesis and Objectives

1.4.1 Rationale

The relationship between FoxO transcription factors and several life-threatening diseases – most notably cancer and diabetes – have encouraged many researchers to mark FoxO proteins as potential therapeutic targets (Yang and Hung 2009; Lu and Huang 2011). Indeed, novel FoxO1 inhibitors are currently being developed and tested in mice (Nagashima et al. 2010; Tanaka et al. 2010). Therefore, searching for methods or agents able to modulate FoxO activity would be of significance for future clinical developments

As detailed above, FoxO protein activity is tightly regulated by oxidative stress. GSH is known for its ability to regulate the cellular redox state and to reflect the level of oxidative stress. In addition, recent data show that FoxO4 contains a reactive cysteine residue at position 477 that is susceptible to transient oxidation to form an intermolecular disulfide link to other proteins (Dansen et al. 2009). Based on these findings, we predicted that glutathione may be involved in FoxO S-glutathiolation, and subsequently regulation of FoxO activity. Using agents that can deplete glutathione levels, such as diethyl maleate (DEM) and buthionine sulfoximine (BSO), we were able to design a set of experiments to investigate the effect of glutathione depletion on insulin-induced FoxO phosphorylation. The selection of these agents was based on their ability

to deplete glutathione effectively in vitro, and the wide use of them in the literature as glutathione-depleting agents.

1.4.2 Hypotheses

- GSH depletion modulates phosphorylation of FoxO transcription factors induced by insulin.
- GSH depletion stimulates stress-responsive signaling, such as cJun N-terminal kinases, which are known modulators of FoxO activity.

1.4.3. Objectives

- To examine FoxO phosphorylation in cells exposed to insulin and the effect of prior glutathione depletion on this phosphorylation.
- To examine the effect of glutathione depletion on the activity of other signaling proteins: the insulin signaling cascade (insulin receptor, Akt, GSK3) and the stress-responsive cJun N-terminal kinases.
- To compare the effects of DEM and BSO regarding FoxO phosphorylation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Diethyl maleate (DEM), L-buthionine sulfoximine (BSO), glutathione ethyl ester (GEE), N-acetyl cysteine (NAC), Ellman's reagent (DTNB), neutral red solution (3.3g/l in PBS), tween 20, sodium dodecyl sulfate (SDS), ammonium persulfate, tetramethylethylenediamine (TEMED) , Dulbecco's modified Eagle's medium (DMEM, 4.5g/l glucose) and non-essential amino acids were purchased from Sigma Aldrich (St. Louis, MO, USA). Bio-Rad Laboratories (Hercules, CA, USA) were the source for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) apparatus and acrylamide. Glycerol and hydrochloric acid were purchased from Caledon Labs (Halton Hills, ON, Canada). Tris hydroxymethyl aminomethane (TRIS), penicillin/ streptomycin and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). Glycine and sodium hydrochloride were purchased from Fisher Scientific (Toronto, ON, Canada). Okadaic acid and Calyculin A were purchased from Enzo Life Sciences (Burlington, ON, Canada). Leptomycin B was purchased from Alexis Biochemicals (Burlington, ON, Canada). All primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) except anti-β-actin, which was purchased from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were obtained from Dianova (Hamburg, Germany) and GE Healthcare Life Sciences (Baie d'Urfe, QC, Canada), respectively. Fetal calf serum was purchased from GE Healthcare Bio-Sciences Corp (Piscataway Township, NJ, USA). Thermo Scientific (Rockford, Il, USA) was the source for Supersignal west pico chemiluminescent substrate and Supersignal west femto chemiluminescent substrate for western blot signal detection; and bicinchoninic acid (BCA) protein assay kit for protein estimation.

2.2 Methods

2.2.1 Cell culture

HepG2 human hepatic carcinoma cells were used. This is because FoxO1 and FoxO3 are highly expressed in this cell line. HepG2 cells were grown to approximately 70-80% confluence prior to treatment in all experiments except in BSO experiments, in which they were grown to 65-70% confluence prior to treatment. This was to avoid over-confluence after the long treatment time with BSO. Dulbecco's modified Eagle's medium (DMEM, 4.5g/l glucose) with 10% (v/v) fetal calf serum, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin/streptomycin (100x mix, 10,000 units pen and 10mg strep/ml) were used to grow cells at 37 °C with 5% (v/v) CO_2. Trypsin 0.5% (5g/l) was used to help detach cells before splitting.

2.2.2 Neutral red cytotoxicity assay

Neutral red is a weak cationic dye able to penetrate the cytoplasmic membrane of living cells and bind to anionic sites in the lysosomal matrix (Repetto et al. 2008). Upon exposure of cells to toxic stimuli, lysosomal fragility results in decreased binding of neutral red. In our experiments, cells were grown in 24-well plates and incubated with serum free working solutions containing different concentrations of DEM or BSO in quadruplicates, i.e. four wells incubated with the same concentration for 1-hour and 2-hour periods with DEM and for 24-hour and 48-hour periods with BSO. Following incubation, treatment solutions were removed and cells were washed once with phosphate buffered saline (PBS) and incubated with neutral red solution (diluted 1:50 in serum-free media) for 2 hours at 37 °C, followed by washing with PBS and the addition of 500 µl of fixing solution containing ethanol:acetic acid:water (50:1:49) for extraction

of neutral red from viable cells. Extracts were transferred to 96-well plates for reading absorption at 550 nm, background reading at 405 nm. Data were plotted using standard viability curve.

2.2.3 Thiol determination

Cells were grown in 10 cm cell culture plates prior to treatment then lysed with 250 µl cold 0.01N HCL and stored at -80 °C for no longer than 1 week prior to thiol determination. The agent used to determine thiol levels was dithionitrobenzoic acid (DTNB), also known as Ellman's reagent (Ellman 1958). In the presence of thiols, DTNB is reduced to mixed disulfides and thionitrobenzoate (TNB) which can be measured spectrophotometrically at 412 nm. At the day of the assay, lysates were thawed on ice, vortexed then centrifuged at 4°C, 14,000 rpm for 10 minutes. Supernatants were collected and kept on ice, then deproteinized by addition of cold 20% (w/v) 5-sulfosalicylic acid (SSA) in a ratio of 68 ul to 200 ul of sample, vortexed then incubated on ice for 5 min followed by centrifugation for 5 min at 4°C, 14,000 rpm. Samples afterwards were treated with reaction buffer containing DTNB in 1M Tris, pH 8. For every 10 samples, 500 ul DTNB + 9 ml Tris reaction buffer was prepared. 50 ul of sample was mixed with 950 ul of reaction buffer. Original DTNB stock was 50 mM in 95% ethanol. After mixing with reaction buffer, samples and standards were left for 5 minutes before taking absorption at 412 nm. N-acetyl cysteine was selected as a thiol standard treated similarly to the samples of interest throughout the assay. A standard curve was obtained in comparison to which thiol levels of samples were determined.

2.2.4 Protein determination

Bicinchoninic acid assay (BCA) was used to estimate protein levels (Smith et al. 1985). The principle of this assay is the formation of Cu^{2+}-protein complex under alkaline conditions, then reduction of Cu^{2+} to Cu^{1+}. The amount of reduction reflects the amount of protein in sample. BCA then chelate with Cu^{1+} forming a purple-colored solution that absorbs light at 562 nm, thus can be measured spectrophotometrically. Bovine serum albumin was selected as a protein standard from which a standard curve was obtained and used to estimate sample protein levels. The same lysates that were assayed for thiol determination were assayed for protein levels in order to determine the ratio of thiol to protein for each sample.

2.2.5 Western blot

Cells were grown in 6-well plates, treated for specific time points, washed with PBS between each treatment, then lysed in 2x Laemmli buffer (125 mM Tris/HCl pH 6.8, 4% (w/v) SDS, 20% glycerol, 100 mM dithiothreitol and traces of bromophenol blue) after treatment on ice. Samples were then stored at -20 °C. At the day of the experiment, samples were sonicated for 2 seconds, centrifuged for 10 minutes, heated at 95 °C for 5 minutes, then centrifuged briefly. Equal amount from each sample was loaded into SDS-polyacrylamide gels (10% w/v) for electrophoresis and blotting. Tank blotting and nitrocellulose membranes were used for transferring proteins from SDS gels. Membranes were then blocked with 5% (w/v) non-fat milk for 1 hour on shaker. Afterwards, Immunodetection was performed using the following antibodies: anti-phospho-Akt (S473) 1:2000 dilution, anti-phospho-FoxO1a/FoxO3a (T24/T32) 1:2000 dilution, anti-Akt and anti-FoxO1 1:2500 dilution and anti-β-Actin 1:10000 dilution. All primary antibodies were

incubated on membranes overnight at 4 °C while shaking, except β-actin which was incubated for 1 hour at room temperature. Primary antibodies were removed and membranes washed three times with Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST), 10 minutes for each wash on shaker. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were incubated on membranes for 1 hour at room temperature, followed by TBST washing three times, 10 minutes for each wash. Afterwards, super signal solution was added to the membranes for few minutes before film exposure. All antibodies were diluted in 5% (w/v) non-fat milk powder in TBST before incubation. Data analysis was performed using ImageJ free software.

2.2.6 Statistical analysis

Data in this work are presented as means ± standard error of the mean except thiol per protein and BSO cytotoxicity data, which were presented as means ± min/max values. Control and treated group measurements were compared using One-way ANOVA followed by Tukey post-hoc test. A result was considered statistically significant where $p < 0.05$. SPSS software was used to perform statistical analysis and Image J software was used to evaluate western blot films.

CHAPTER 3

RESULTS

3.1 Effect of diethyl maleate and buthionine sulfoximine on cell viability

HepG2 cells were exposed to different concentrations of DEM and BSO for multiple time points. These were selected based on available literature and our research objective. DEM concentrations were 1, 3 and 10 mM, and incubation was for 1 hour and 2 hours. BSO concentrations were 0.3, 1 and 3 mM, and exposure for 24 hours and 48 hours. The longer incubation with BSO was due to the fact that this agent inhibits glutathione synthesis rather than deplete glutathione directly. All treatments were performed in serum-free media. DEM showed no significant toxicity in the 1-hour time point with all concentrations, while in the 2-hour time point 10 mM DEM was associated with significant percentage of cell death compared to control. DEM experiments were performed 3 independent times. BSO showed no toxicity with all concentrations used at both time points. BSO experiments were performed 2 independent times. Results are shown in Figure 3.1.

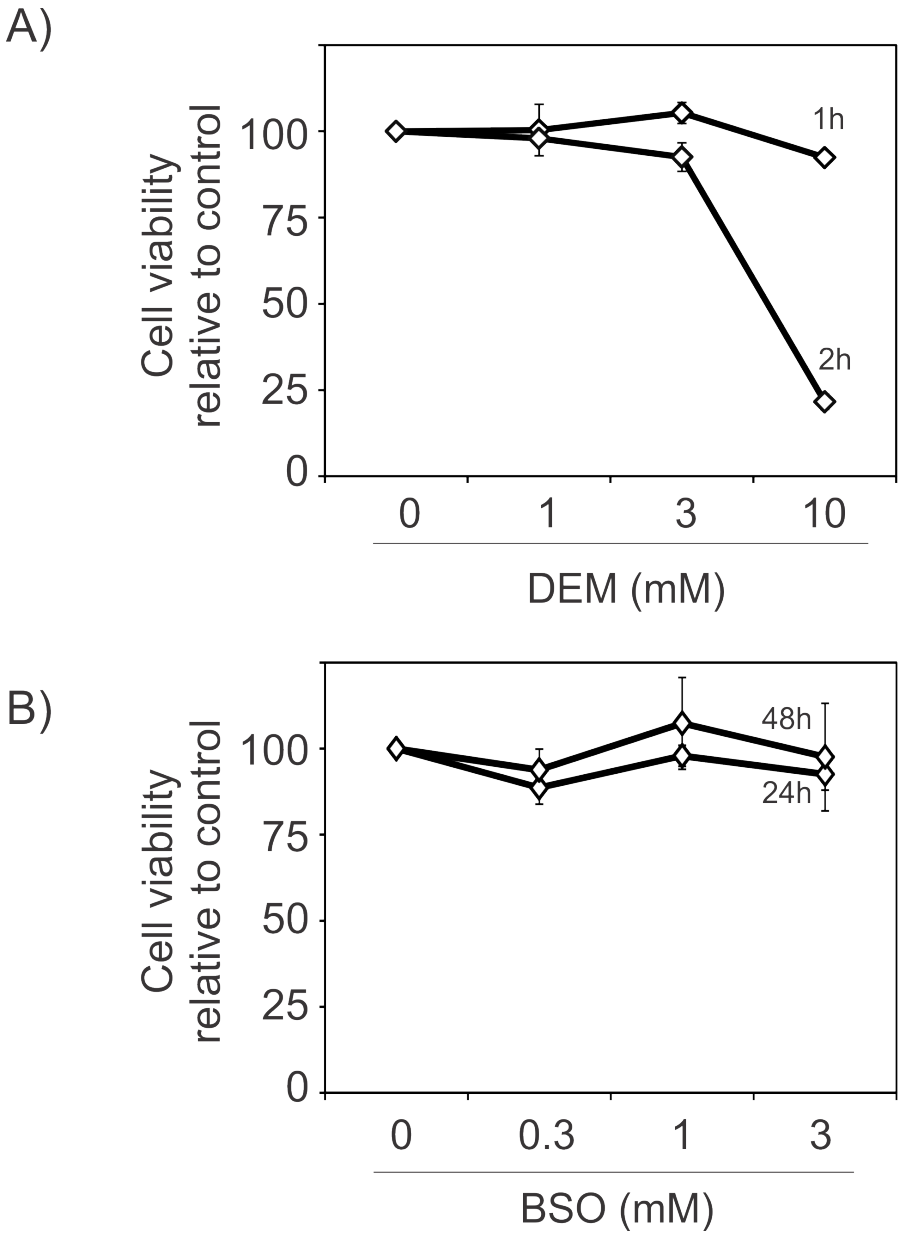

Figure 3.1 Effect of DEM and BSO on cell viability. A) Cell viability after 1-hour and 2-hour incubations with different concentrations of DEM relative to control. Data are presented as means ± SE (n = 3). **B)** Cell viability after 24-hour and 48-hour incubations with different concentrations of BSO relative to control. Data are presented as means ± min/max values (n = 2).

3.2 Effect of diethyl maleate and buthionine sulfoximine on thiol levels

To assess the effectiveness of DEM and BSO in lowering thiol levels, thiol measurements were performed using the DTNB assay. A BCA assay for protein content estimation was performed with the same lysates to normalize the total thiol concentrations to protein content. Albumin was used as a protein standard and N-acetyl cysteine was used as a thiol standard. Incubation with different concentrations of DEM showed significant concentration-dependent depletion of thiols after 1-hour and 2-hour treatments. On the other hand, incubation with different concentrations of BSO showed almost equal depletion of thiols in all concentrations but slightly stronger thiol depletion with 48-hour incubation. All treatments were performed in serum-free media. Results are shown in Figure 3.2.

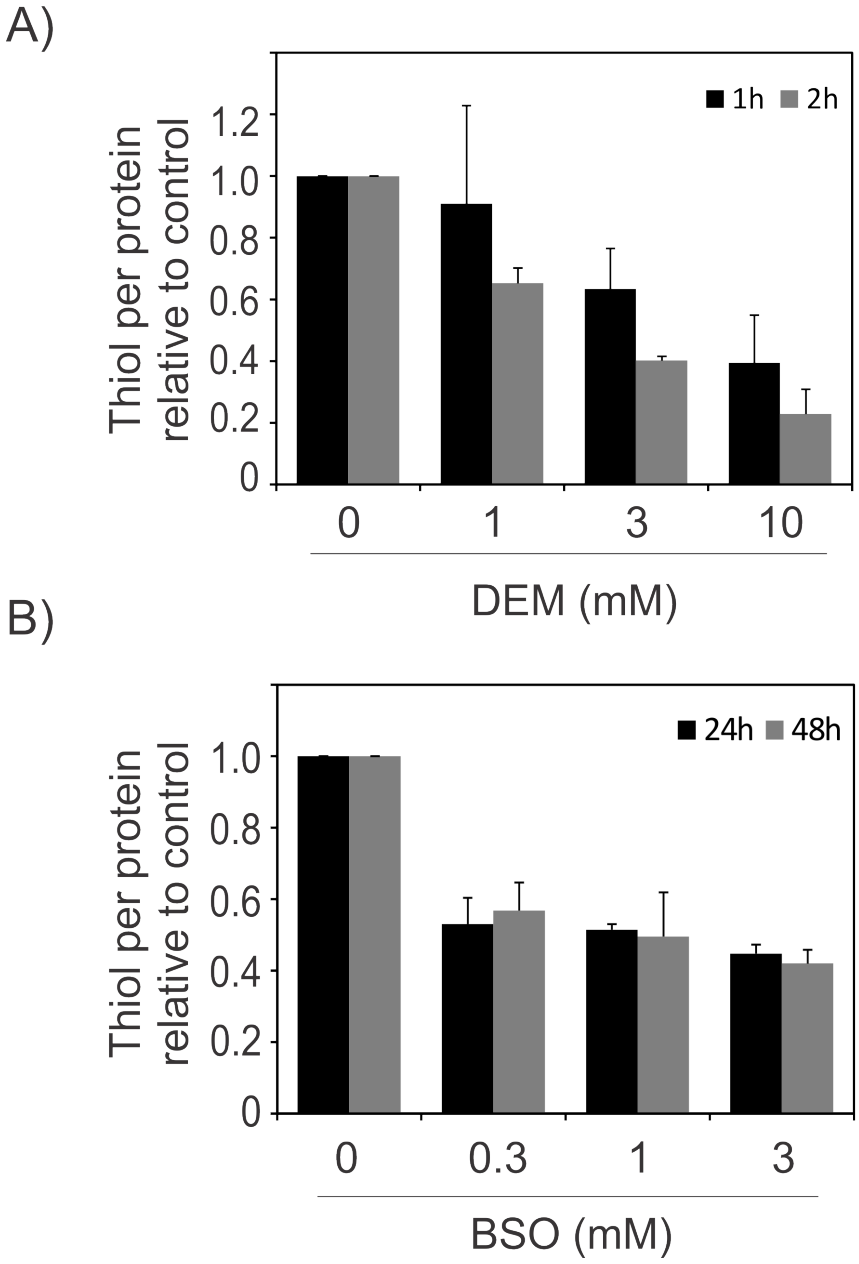

Figure 3.2 Effect of DEM and BSO on thiol levels. A) Thiol per protein levels relative to control after 1-hour and 2-hour incubation with different concentrations of DEM. **B)** Thiol per protein levels relative to control after 24-hour and 48-hour incubation with different concentrations of BSO. Data are presented as means ± min/max value (n = 2).

3.3 Effect of diethyl maleate on FoxO signaling

DEM is a frequently used agent for the study of stress-induced effects. It can deplete glutathione in a reaction catalyzed by glutathione-S-transferases (Adams et al. 1983). However, in addition to glutathione depletion, DEM can conjugate and deplete other intracellular thiols due to its strong electrophilic properties (Bannai 1984; Aoshiba et al. 1999). We investigated the effect of DEM treatment on FoxO transcription factors as well as other proteins in the insulin signaling cascade.

3.3.1 FoxO phosphorylation

To study the effect of DEM on FoxO signaling, insulin was used as a stimulator of FoxO phosphorylation by activating Akt which in turn phosphorylate FoxO at sites known to cause FoxO cytoplasmic localization and inactivation (Thr24 for FoxO1 and Thr32 for FoxO3a). HepG2 cells were incubated with different concentrations of DEM for 1 hour and 2 hours followed by 100 nM insulin incubation for 30 minutes. All incubations were performed in serum free media to avoid interference of growth factors with FoxO signaling as they are known to affect FoxO localization and activity. DEM appears to block insulin-induced FoxO phosphorylation in a concentration-dependent and time-dependent manner. 3 and 10 mM DEM incubation for 1 hour significantly blocked FoxO phosphorylation after insulin treatment compared to control, whereas 3 mM DEM incubation for 2 hours more significantly blocked insulin-induced FoxO phosphorylation. Results are shown in Figure 3.3.

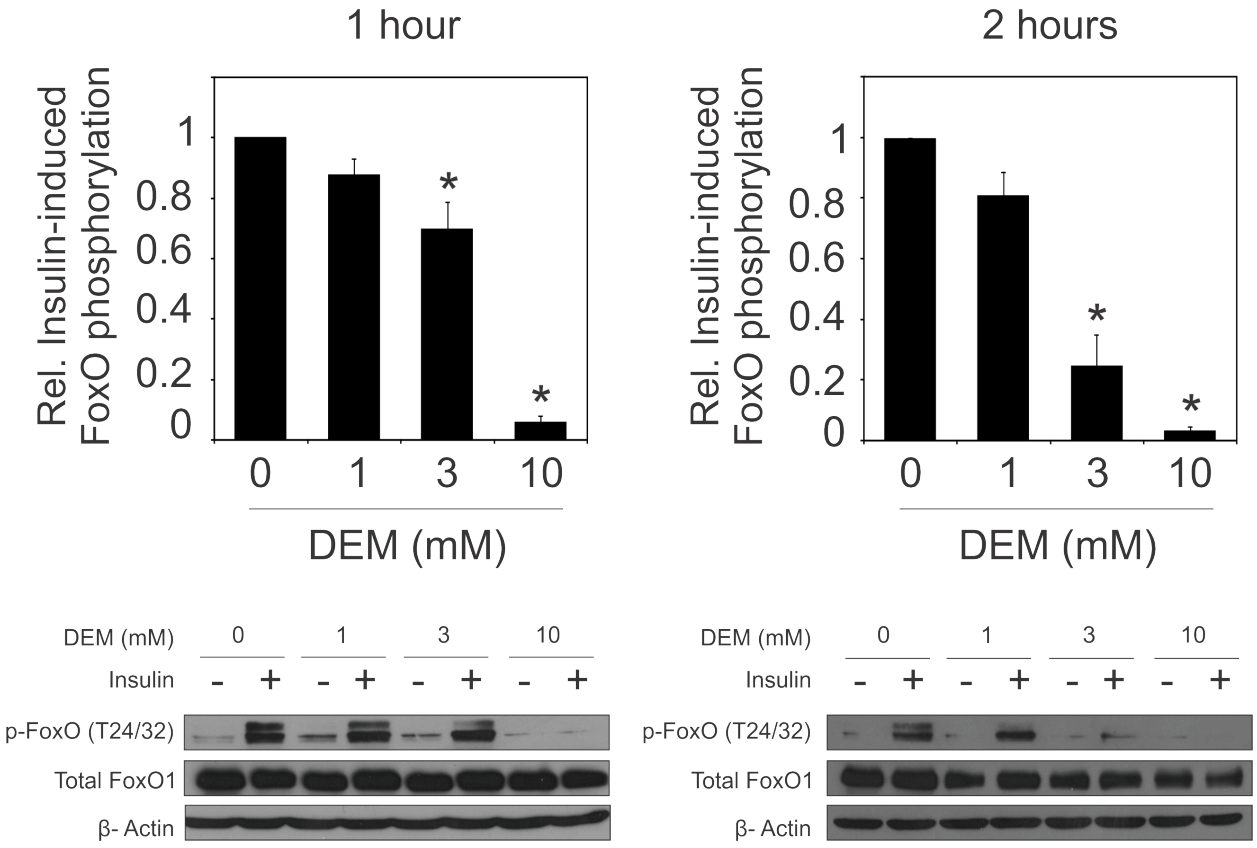

Figure 3.3 Effect of DEM on insulin-induced FoxO1/3a (Thr24/Thr32) phosphorylation. FoxO1/3a phosphorylation as induced by 100 nM insulin treatment for 30 minutes following a 1-hour and 2-hour incubation with different concentrations of DEM. For densitometric analysis, p-FoxO signals were normalized against total FoxO1 levels. Beta actin levels were analyzed to test for alterations in total FoxO1 levels. Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

DEM has been reported to interfere with the nuclear export machinery by inhibiting chromosome region maintenance-1 (Crm1)-dependent export of proteins (Crampton et al. 2009). To investigate if this effect of DEM is responsible for DEM's de-phosphorylating effect on FoxO proteins, HepG2 cells were incubated with 100 nM insulin first for 30 minutes followed by DEM incubation for 30 minutes. This is to allow FoxO proteins to be phosphorylated as a result of insulin stimulation and exiting the nucleus first, then observing DEM's effect on FoxO phosphorylation. DEM still caused significant de-phosphorylation of FoxO even after insulin incubation, indicating that the de-phosphorylating effect of DEM on FoxO is not dependent solely on DEM's effect on nuclear export. This is supported by experiments employed by a known inhibitor of Crm1-dependent nuclear export, leptomycin B (see chapter 3.4). Results are shown in Figure 3.4.

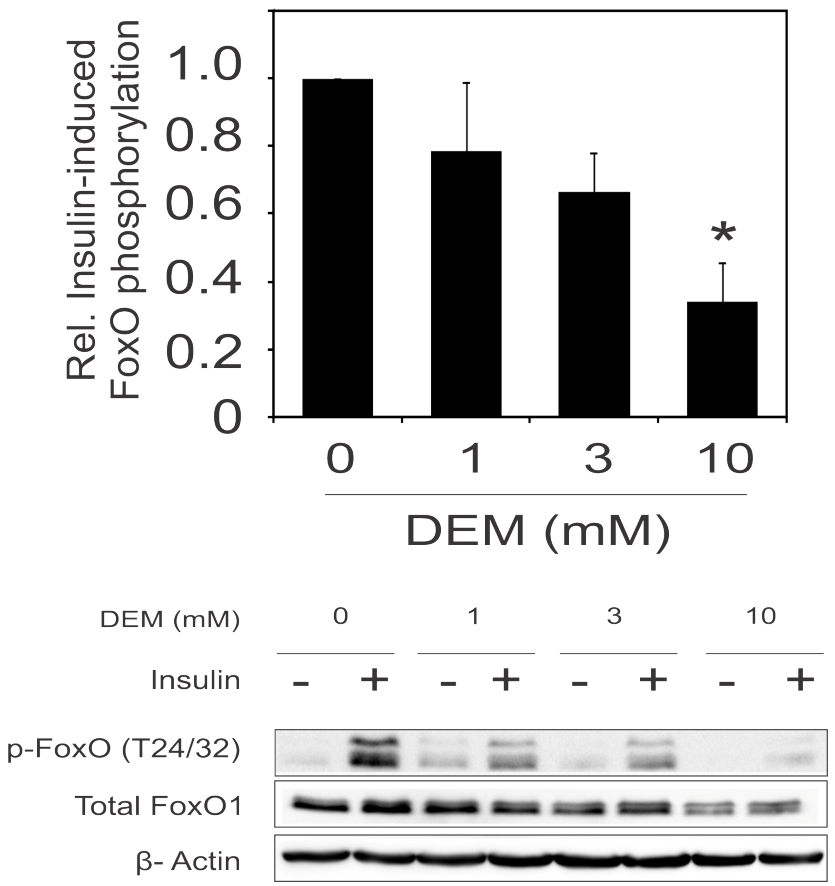

Figure 3.4 Effect of DEM on FoxO1/3a (Thr24/Thr32) phosphorylation post-insulin exposure. FoxO1/3a phosphorylation after a treatment with 100 nM insulin for 30 minutes, followed by 30 minutes of incubation with different concentrations of DEM. For densitometric analysis, p-FoxO signals were normalized against total FoxO1 levels. Beta actin levels were analyzed to test for alterations in total FoxO1 levels. Results are presented as means ± SE (n = 4)

* $P < 0.05$ versus control.

3.3.2 Insulin receptor phosphorylation

To investigate the mechanism of the observed DEM effect on insulin-induced FoxO phosphorylation, insulin receptor phosphorylation was analyzed. Auto-phosphorylation of tyrosine residues within the insulin receptor indicates its being stimulated. To test whether DEM impairs this early insulin stimulatory effect on insulin receptors, Tyr1150/1151 phosphorylations were investigated. HepG2 cells were incubated with different concentrations of DEM for 1 hour, followed by incubation with 100 nM insulin for 30 minutes. DEM at 10 mM significantly blocked insulin receptor phosphorylation. This indicates that DEM has an impairing effect on insulin signaling already at the level of the insulin receptor by interfering with insulin receptor tyrosine auto-phosphorylation. Results are shown in Figure 3.5.

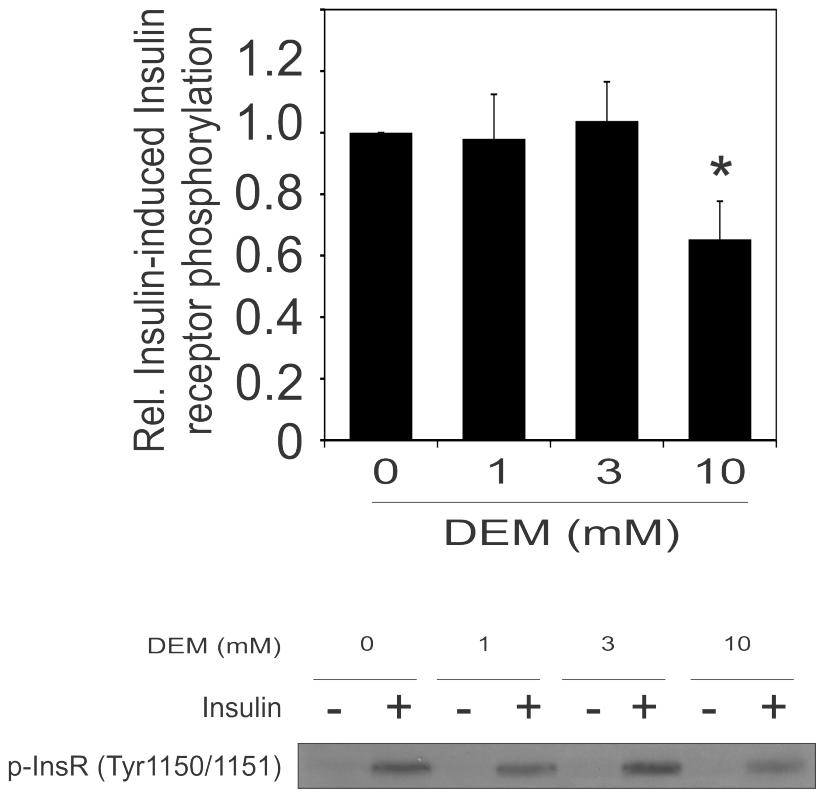

Figure 3.5 Effect of DEM on insulin-induced receptor (Tyr1150/1151) phosphorylation. Insulin receptor tyrosine phosphorylation after a 1-hour incubation with different concentrations of DEM followed by 30 minutes treatment with 100 nM insulin. Densitometric analysis of insulin receptor phosphorylation at (Tyr1150/1151) of insulin-treated samples are shown. Beta actin levels were analyzed and no changes were observed (blots not shown, refer to Figure 3.3). Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

3.3.3 Akt phosphorylation

Akt (protein kinase B) is known to be activated by insulin stimulation. This activated Akt is responsible for FoxO phosphorylation at sites of interest (Thr24 and Thr32 in FoxO1 and FoxO3a respectively). Therefore, Akt phosphorylation at Ser473 was investigated. HepG2 cells were incubated with different concentrations of DEM for 1 hour and 2 hours, followed by incubation with 100 nM insulin for 30 minutes. Following a 1-hour DEM incubation with 30 minutes insulin stimulation thereafter, no significant change in insulin-induced Akt phosphorylation was observed at all concentrations. However a slight trend of reduction in Akt phosphorylation with 10 mM DEM was observed. Moreover, following a 2-hour DEM incubation with 30 minutes of insulin stimulation thereafter, there was a significant attenuation of insulin-induced Akt phosphorylation with 10 mM DEM. This indicates that DEM interferes with insulin signaling by impairing Akt phosphorylation only at high concentrations. Results are shown in Figure 3.6.

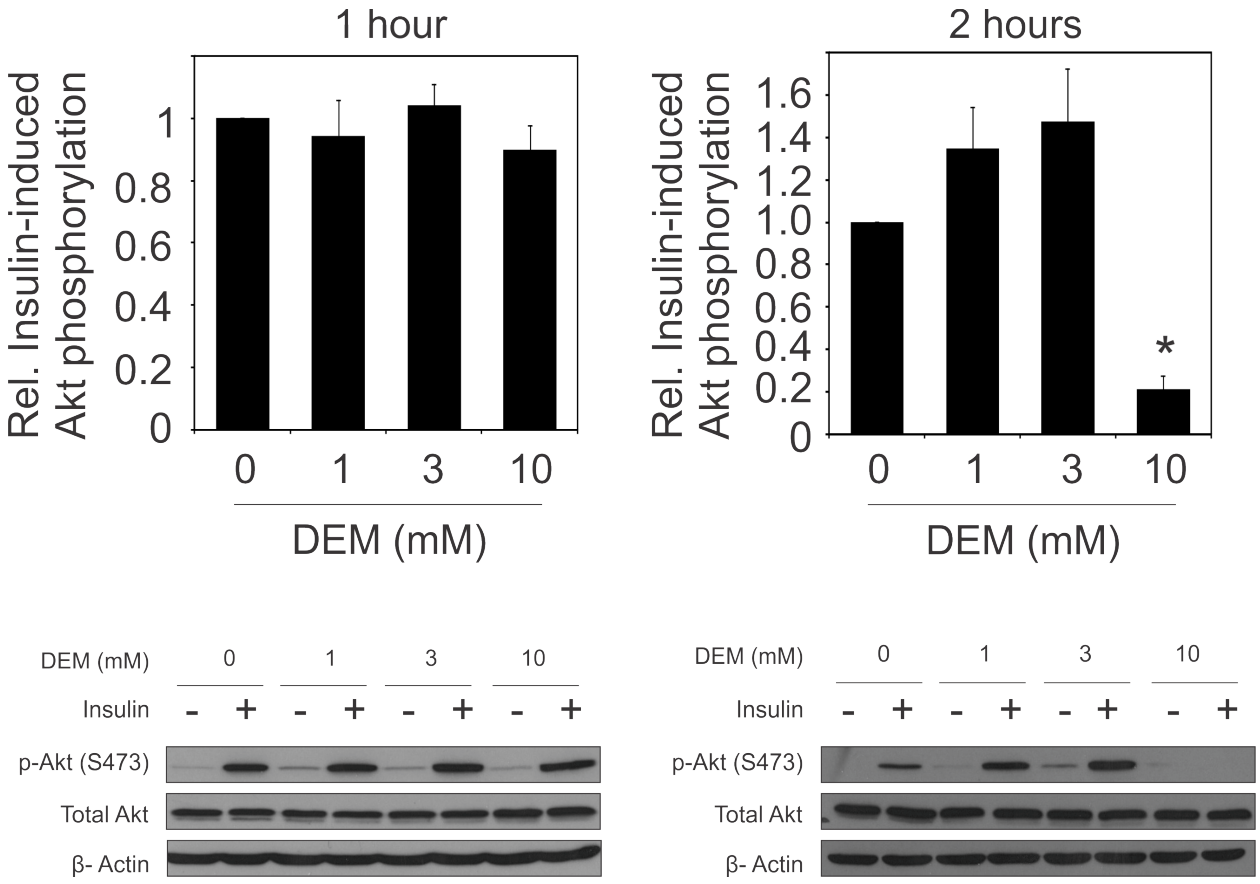

Figure 3.6 Effect of DEM on insulin-induced Akt (Ser473) phosphorylation. Akt phosphorylation following 1-hour and 2-hour incubation with different concentrations of DEM followed by 100 nM insulin treatment for 30 minutes. For densitometric analysis, p-Akt signals were normalized against total Akt levels. Beta actin levels were analyzed to test for alterations in total Akt levels. Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

3.3.4 GSK-3 alpha phosphorylation

Glycogen synthase kinase 3 alpha (GSK-3α) is a known substrate of Akt. Akt activation by insulin signaling or growth factors leads to GSK phosphorylation at Ser21 and subsequent inhibition of activity. To further confirm the interference of DEM with insulin signaling, we investigated GSK-3α phosphorylation at Ser21. HepG2 cells were incubated with multiple concentrations of DEM for 1 hour followed by 100 nM insulin incubation for 30 minutes. Following incubation with 10 mM DEM, insulin-induced GSK-3α phosphorylation was significantly blocked. This indicates that DEM interferes with insulin signaling also through impairing insulin-induced GSK-3α phosphorylation. Results are shown in Figure 3.7.

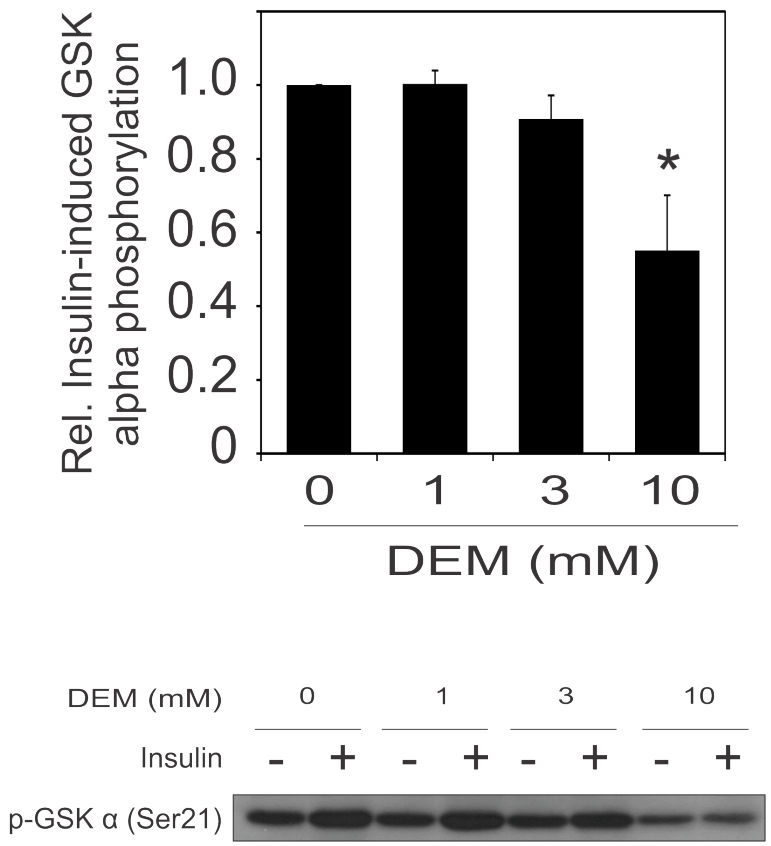

Figure 3.7 Effect of DEM on insulin-induced GSK-3 alpha (Ser21) phosphorylation. GSK-3α phosphorylation following a 1-hour incubation with different concentrations of DEM and 30 minutes of treatment with 100 nM insulin thereafter. Densitometric analysis of GSK3-alpha phosphorylation at (Ser21) of insulin-treated samples are shown. Beta actin levels were analyzed and no changes were observed (blots not shown, refer to Figure 3.3). Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

3.3.5 SAPK/JNK phosphorylation

Stress-activated protein kinases (SAPK) include the c-Jun N-terminal kinases (JNK) and are members of the MAPK family. Their activation is known to reflect a cellular response to stress. We were interested in whether DEM affects JNK activity and therefore analyzed JNK phosphorylation at Thr183/Tyr185. DEM is a known inducer of cellular stress which is a strong regulator of FoxO. HepG2 cells were incubated with different concentrations of DEM for 1 hour, followed by 100 nM insulin incubation for 30 minutes. DEM at 3 and 10 mM was associated with significant activation of JNK regardless of insulin treatment. This indicates that DEM is causing strong cellular stress. Results are shown in Figure 3.8.

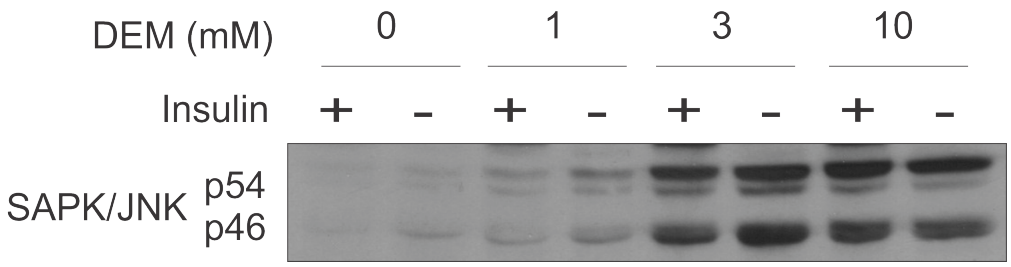

Figure 3.8 Effect of DEM on SAPK/JNK (Thr183/Tyr185) phosphorylation. SAPK/JNK phosphorylation following 1-hour incubation with different concentrations of DEM followed by 30 minutes incubation with 100 nM insulin where indicated. The blot is representative of three independent experiments with similar results.

3.3.6 FoxO phosphorylation in the presence of phosphatase inhibitors

Based on the results shown in previous sections, the observed DEM effect on insulin-induced FoxO phosphorylation could be due to DEM induction of cellular phosphatases. Indeed, DEM has been reported to generate ROS which have been suggested to induce cellular phosphatases (Esposito et al. 2000). In addition, FoxO activity has been shown to be regulated by the Ser/Thr phosphatase PP2A (Yan et al. 2008). Therefore, DEM co-incubation with phosphatase inhibitors was performed to investigate a possible correlation between cellular phosphatases and the observed DEM effect on insulin-induced FoxO phosphorylation. Okadaic acid (OA) and Calyculin A (CA), potent PP2A and PP1 inhibitors were used at effective concentrations able to increase FoxO basal phosphorylation at Thr24/Thr32 (Figure 3.9 A, B). HepG2 cells were incubated with 10 mM DEM + 250 nM OA or 15 nM CA for 1 hour, followed by 30 minutes of insulin treatment. Interestingly, co-incubation with OA or CA did not affect the effect of DEM on insulin-induced FoxO phosphorylation. Results are shown in Figure 3.9.

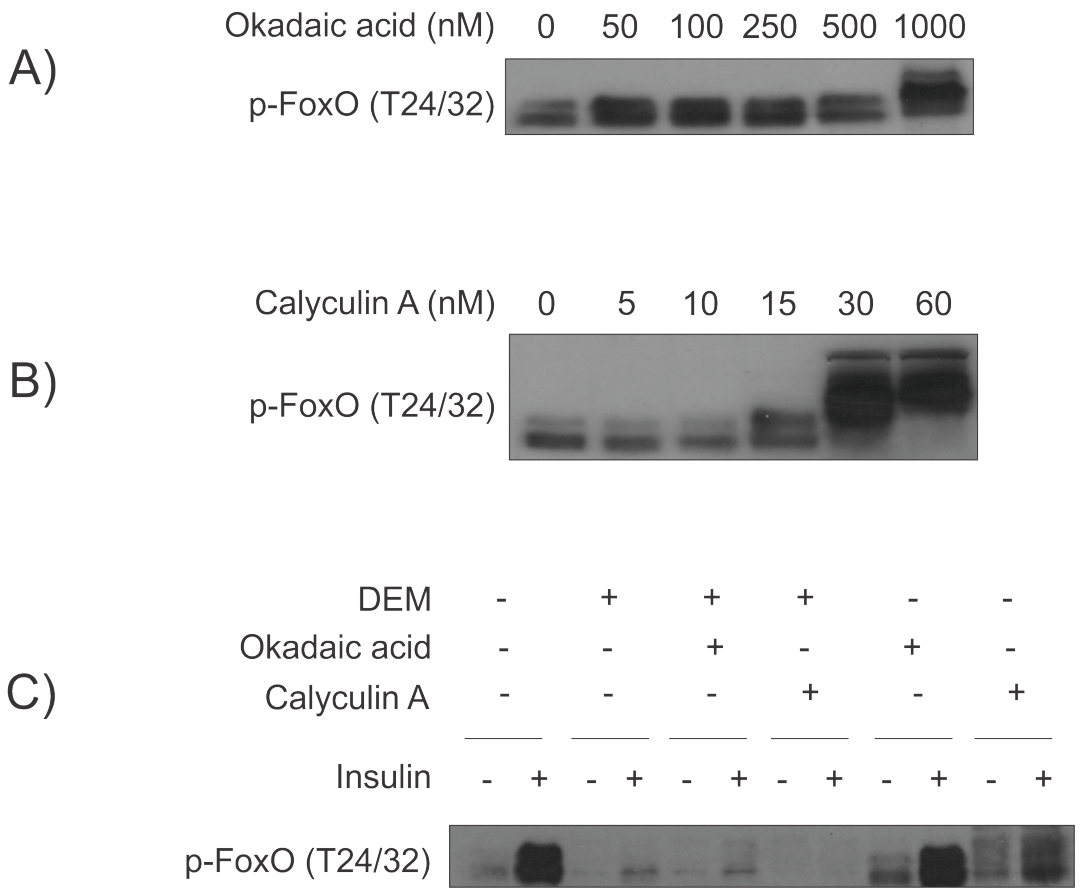

Figure 3.9 Effect of phosphatase inhibitors on FoxO phosphorylation following DEM and insulin exposure. A) Basal FoxO phosphorylation following 1-hour incubation with different concentrations of okadaic acid. (n = 2) **B)** Basal FoxO phosphorylation following 1-hour incubation with different concentrations of calyculin A (n = 2). **C)** FoxO phosphorylation following 1-hour of 10 mM DEM co-incubation with 250 nM okadaic acid or 15 nM calyculin A, followed by 100 nM of 30 minutes insulin treatment. (n = 3). Beta actin levels were analyzed and no changes were observed (blots not shown).

3.3.7 FoxO and Akt phosphorylation in the presence of glutathione

To examine whether the effect of DEM on FoxO signaling is related to glutathione depletion caused by DEM, membrane-permeable glutathione ester was co-incubated with DEM to restore or replenish intracellular glutathione levels. Insulin-induced Akt and FoxO phosphorylations were checked. HepG2 cells were incubated with 10 mM DEM and 10 mM glutathione ethyl ester (GEE) for 1 hour followed by 100 nM insulin treatment for 30 minutes. In addition, non-membrane permeable reduced glutathione (GSH) was also co-incubated with DEM at the same concentration (10 mM) to check for direct interaction between DEM and GSH outside cells. Interestingly, the presence of GSH or GEE significantly reversed the effect of DEM on insulin-induced FoxO phosphorylation indicating a possible direct interaction between DEM and GSH/ GEE (Figure 3.10). To further confirm this finding, DEM and GSH were mixed in test tubes and thiol determination was performed. A significant decrease in thiols was observed upon mixing the agents, suggesting a direct interaction of DEM with thiols (data not shown). This indicates that DEM is a strong electrophile that can react with GSH even in the absence of glutathione-S-transferases.

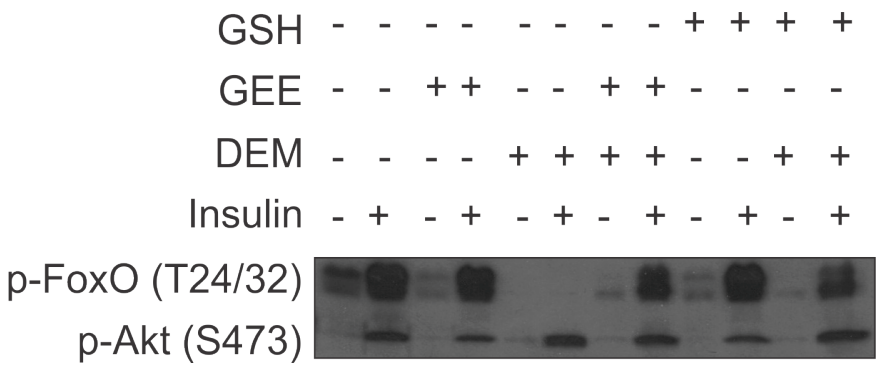

Figure 3.10 Effect of DEM on Akt (Ser473) and FoxO phosphorylation (Thr24/Thr32) in the presence of glutathione. FoxO and Akt phosphorylation following 1-hour co-incubation of 10 mM DEM with 10 mM GEE or 10 mM GSH followed by 30 minutes incubation with 100 nM insulin. (n = 2). The effect of DEM on insulin-induced FoxO phosphorylation was reversed in the presence of either GSH or GEE.

3.4 Effect of leptomycin B on FoxO signaling

Leptomycin B (LMB) is a known specific nuclear export inhibitor. This is due to LMB's ability to inhibit Crm1/Exportin 1, a protein required for the export of several proteins containing a nuclear export sequence (NES) (Ossareh-Nazari et al. 1997). FoxO proteins contain NES and their nuclear export is dependent on Crm1 activity (Biggs et al. 1999). DEM interferes with Crm1-mediated export of proteins (Crampton et al. 2009). Therefore, to test the possibility that the DEM effect on FoxO phosphorylation is due to DEM's effect on Crm1, LMB was used instead of DEM, following the same treatment protocol. HepG2 cells were incubated with different concentrations of LMB for 1 hour, followed by 100 nM insulin treatment for 30 minutes. Insulin-induced FoxO and Akt phosphorylation were investigated. Interestingly, both insulin-induced FoxO and Akt phosphorylations were unaffected by LMB treatment. This indicates that the observed attenuation of insulin-induced Akt/FoxO phosphorylation by DEM is not dependent on DEM's nuclear localization or its effect on Crm1. Results are shown in Figure 3.11.

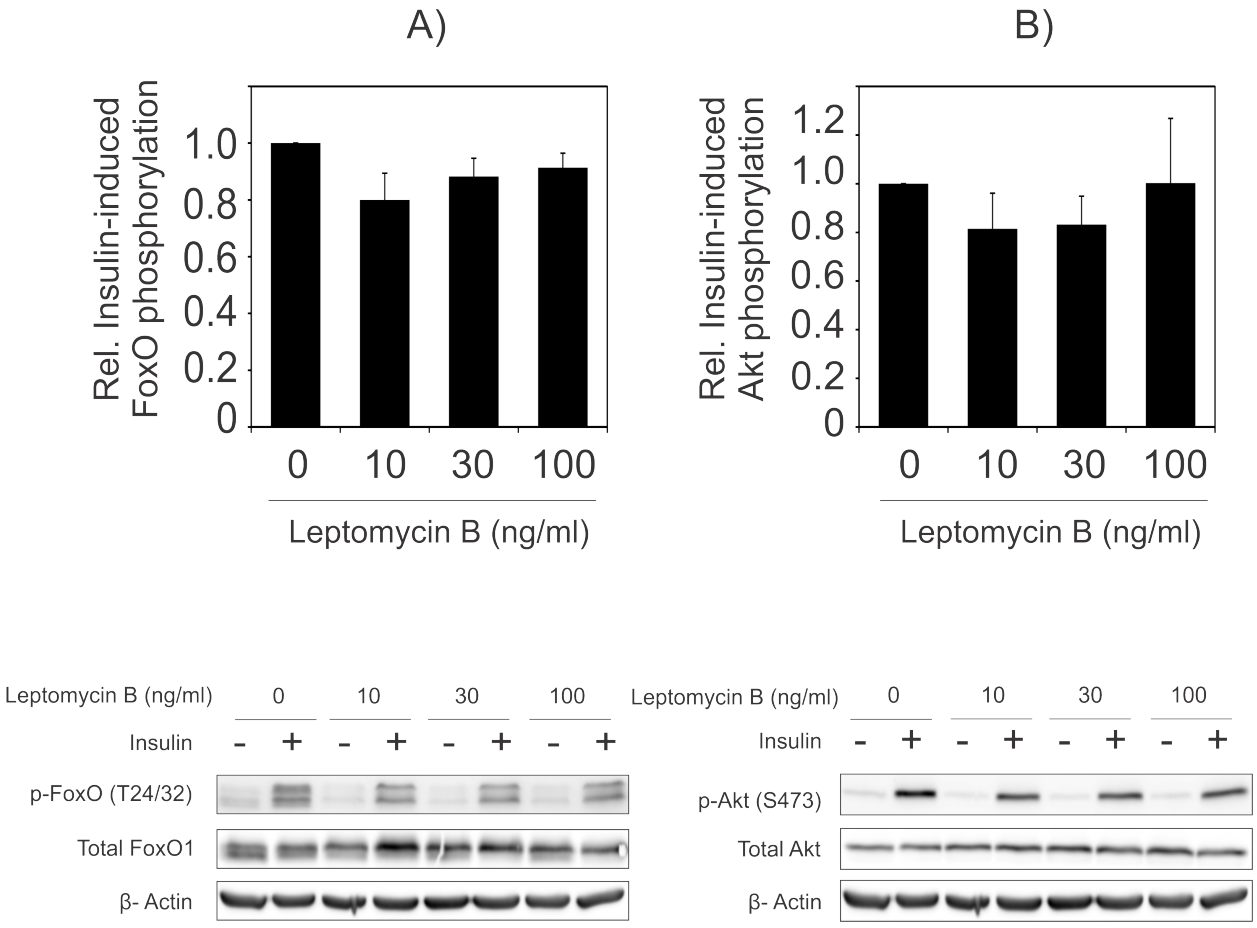

Figure 3.11 Effect of leptomycin B on insulin-induced Akt (Ser473) and FoxO1/3a (Thr24/ Thr32) phosphorylation. A) FoxO1/3a phosphorylation following 1-hour incubation with different concentrations of LMB followed by 100 nM insulin treatment for 30 minutes. **B)** Akt phosphorylation following 1-hour incubation with different concentrations of LMB followed by 100 nM insulin treatment for 30 minutes. For densitometric analysis, p-FoxO signals were normalized against total FoxO1 levels, and p-Akt signals were normalized against total Akt levels. Beta actin levels were analyzed to test for alterations in total FoxO1 and total Akt levels. Results are presented as means ± SE for three independent experiments.

3.5 Effect of buthionine sulfoximine on FoxO signaling

BSO has been known for decades as a potent and specific inhibitor of glutathione synthesis. It directly inhibits gamma-glutamylcysteine synthetase, an enzyme responsible for the first step in the synthesis of glutathione (Griffith and Meister 1979). We here investigated the effect of BSO on insulin-induced phosphorylation of FoxO and other proteins involved in insulin signaling

3.5.1 FoxO phosphorylation

FoxO phosphorylation at Thr24/Thr32 was investigated after after BSO treatment. HepG2 cells were incubated with different concentrations of BSO for 24 hours and 48 hours, followed by incubation with 100 nM insulin for 30 minutes. All incubations were performed in serum free media. No effect on insulin-induced FoxO phosphorylation was observed after a 24-hour incubation with BSO. However, following a 48-hour incubation, BSO at 3 mM attenuated insulin-induced FoxO phosphorylation significantly (Figure 3.12). This indicates that specific depletion of glutathione affects FoxO signaling. Results are shown in Figure 3.12.

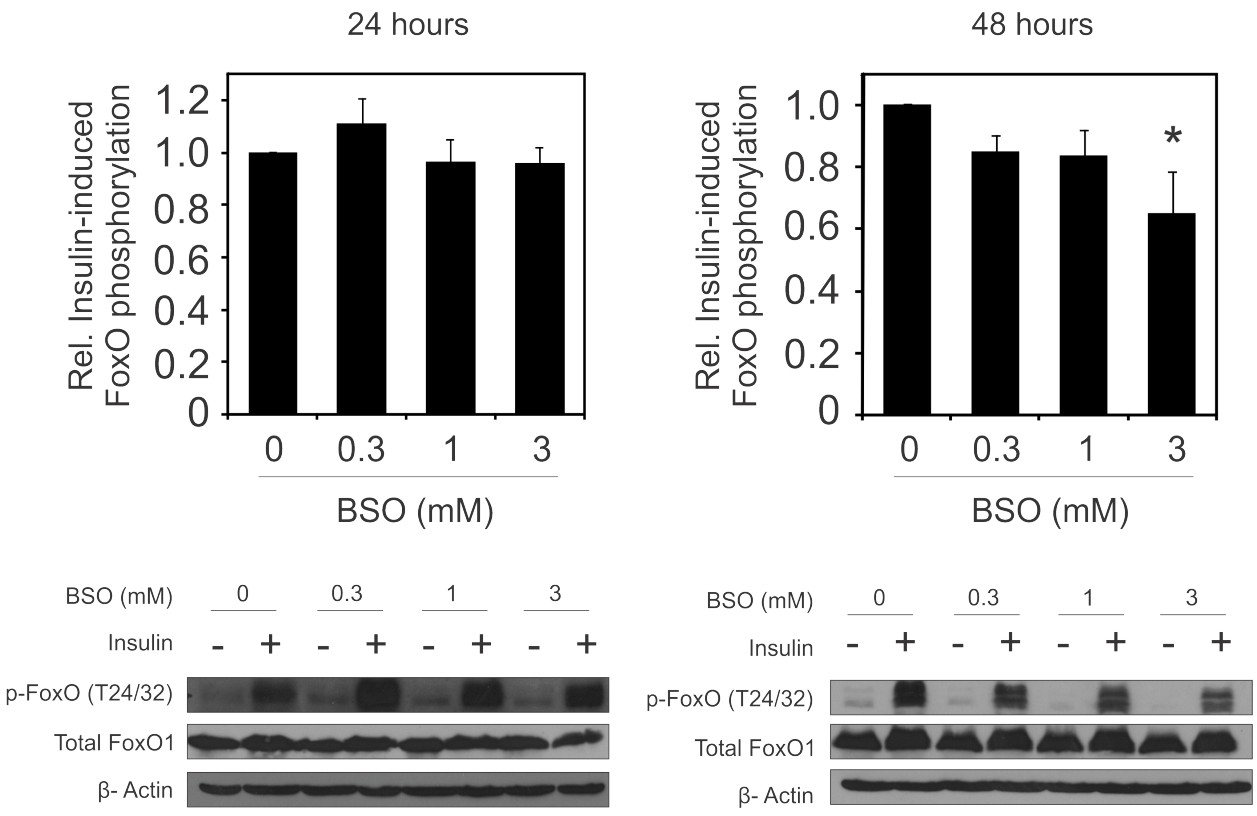

Figure 3.12 Effect of BSO on insulin-induced FoxO1/3a (Thr24/Thr32) phosphorylation. FoxO1/3a phosphorylation was analyzed following a 24-hour and 48-hour incubation with different concentrations of BSO followed by 100 nM insulin treatment for 30 minutes. For densitometric analysis, p-FoxO signals were normalized against total FoxO1 levels. Beta actin levels were analyzed to test for alterations in total FoxO1 levels. Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

3.5.2 Insulin receptor phosphorylation

To investigate the whether BSO affects insulin-induced signaling already upstream of FoxO, insulin receptor phosphorylation was investigated. Auto-phosphorylation of tyrosine residues within the insulin receptor indicates its stimulation. To check this early insulin stimulatory effect on insulin receptors after BSO treatment, Tyr1150/1151 phosphorylation was investigated. HepG2 cells were incubated with 3 mM BSO for 48 hours followed by 5 mM GEE for 3 hours followed by 100 nM insulin incubation for 30 minutes. No significant effect on insulin receptor phosphorylation was observed. However, both GEE and BSO appear to have slightly attenuated insulin receptor phosphorylation. Results are shown in Figure 3.13.

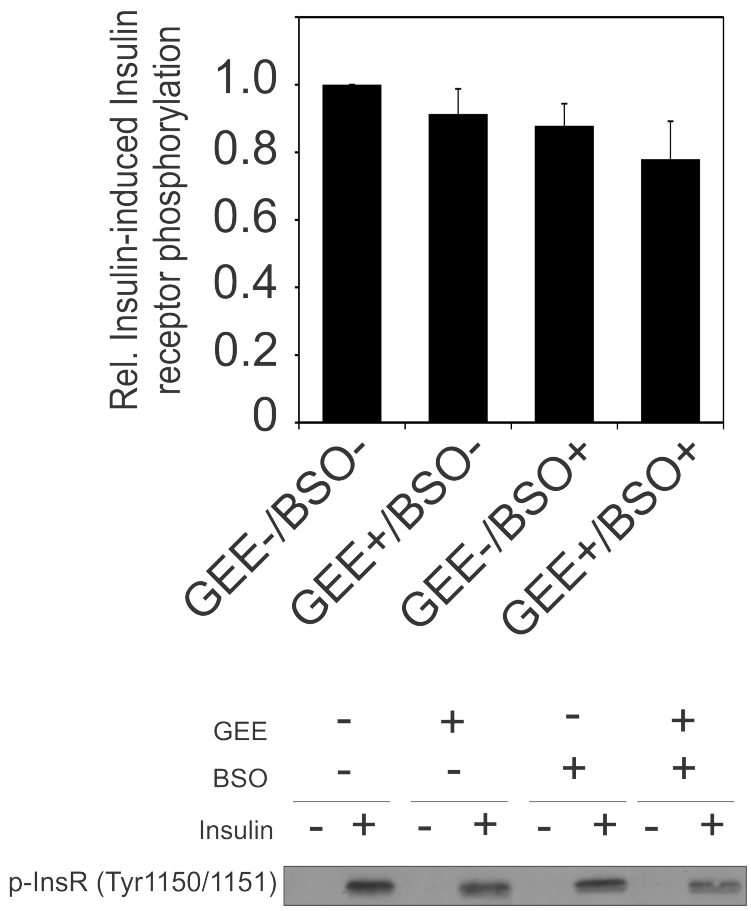

Figure 3.13 Effect of BSO and GEE on insulin-induced receptor (Tyr1150/1151) phosphorylation. Insulin receptor tyrosine phosphorylation following 48-hour incubation with 3 mM BSO followed by 3-hour incubation with 5 mM GEE followed by 30 minutes treatment with 100 nM insulin. Densitometric analysis of insulin receptor phosphorylation at (Tyr1150/1151) of insulin-treated samples are shown. Beta actin levels were analyzed and no changes were observed (blots not shown, see Figure 3.17). Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

3.5.3 Akt phosphorylation

Akt phosphorylation at Ser473 was investigated. HepG2 cells were incubated with different concentrations of BSO for 24 hours and 48 hours followed by 100 nM insulin incubation for 30 minutes. After 24 hours of BSO incubation, no significant change was observed with all concentrations in insulin-induced Akt phosphorylation. However, after 48 hours of BSO incubation there was a significant attenuation of insulin-induced Akt phosphorylation with 3 mM BSO. This indicates that BSO also has an impairing effect on insulin signaling by interfering with insulin-induced Akt phosphorylation. Results are shown in Figure 3.14.

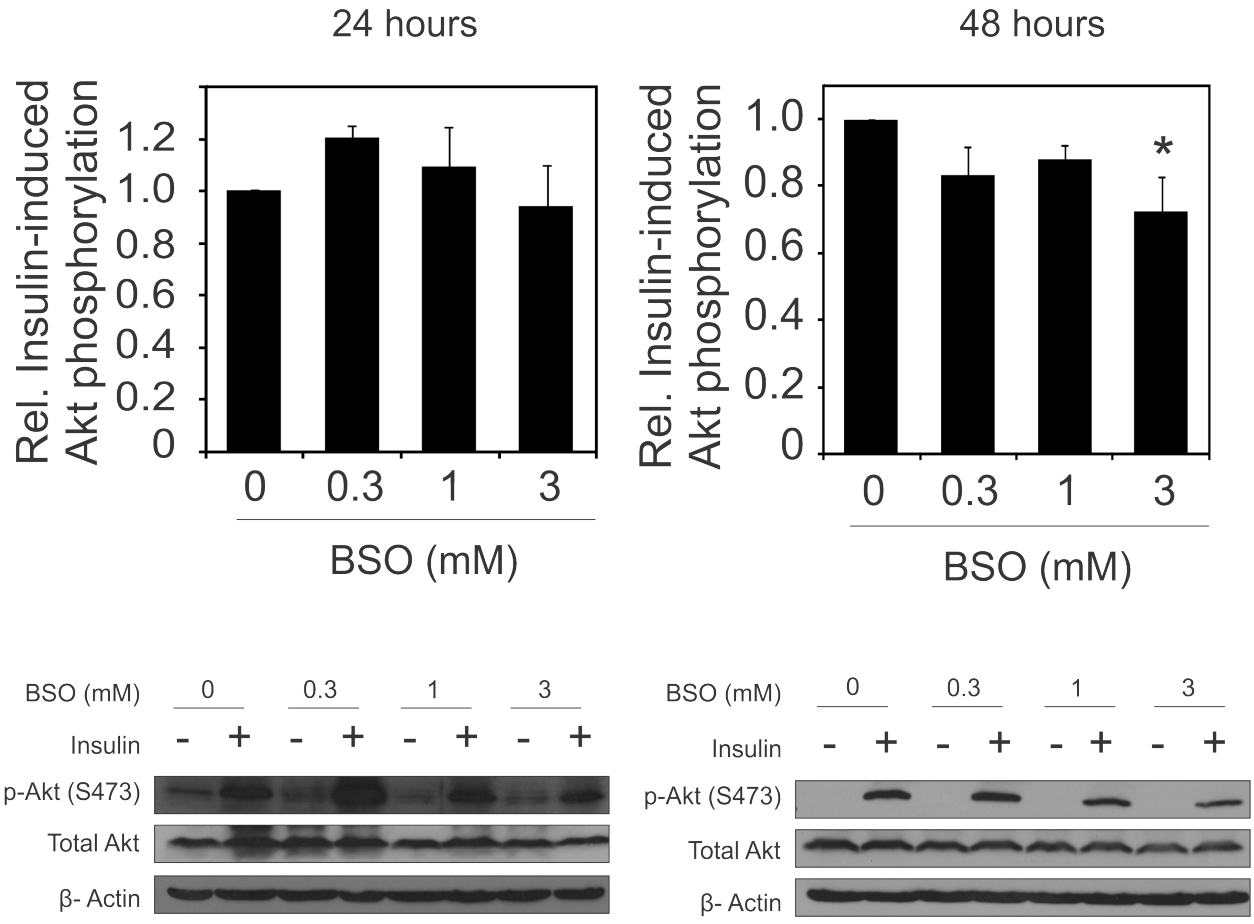

Figure 3.14 Effect of BSO on insulin-induced Akt (Ser473) phosphorylation. Akt phosphorylation following 24-hour and 48-hour incubation with different concentrations of BSO followed by 100 nM insulin treatment for 30 minutes. For densitometric analysis, p-Akt signals were normalized against total Akt levels. Beta actin levels were analyzed to test for alterations in total Akt levels. Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

3.5.4 GSK-3 alpha phosphorylation

To further confirm the effect of BSO on insulin signaling, we investigated a downstream target of Akt, GSK-3α phosphorylation at Ser21. HepG2 cells were incubated with 3 mM BSO for 48 hours, followed by addition of 5 mM GEE for 3 hours in presence of BSO, followed by washing with PBS then incubating with 100 nM insulin for 30 minutes. No significant change was observed with insulin-induced GSK-3α phosphorylation. However, a slight inhibition trend of GSK-3α phosphorylation was evident with BSO treatment. Results are shown in Figure 3.15.

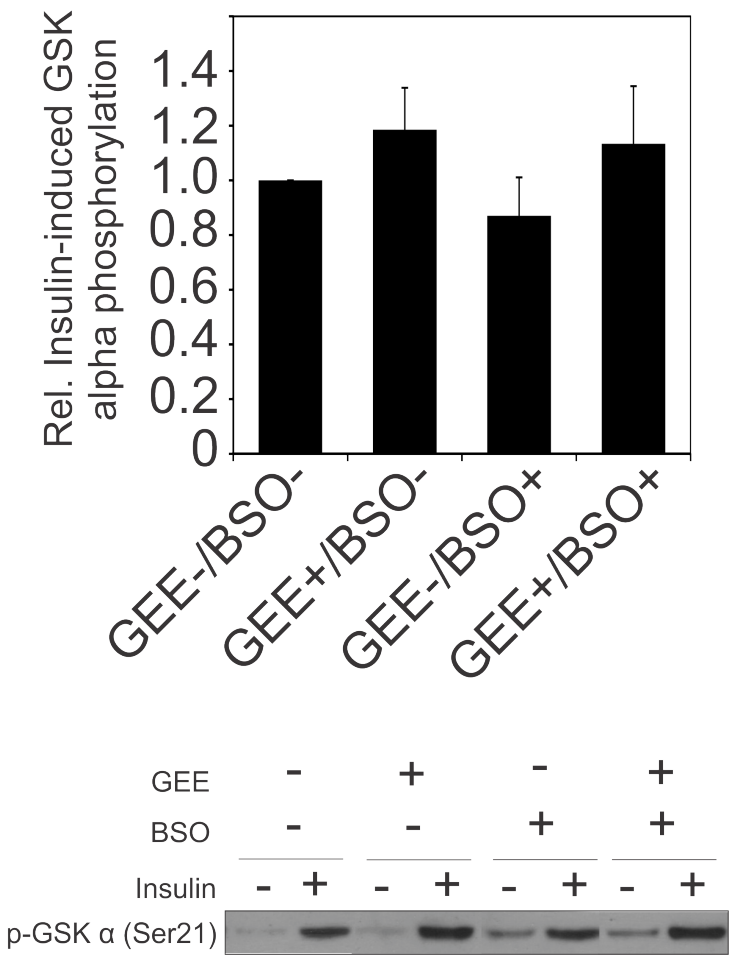

Figure 3.15 Effect of BSO on insulin-induced GSK-3 alpha (Ser21) phosphorylation. GSK-3α phosphorylation following 48-hour incubation with 3 mM BSO followed by 3-hour incubation with 5 mM GEE followed by 30 minutes treatment with 100 nM insulin. Densitometric analysis of GSK3-alpha phosphorylation at (Ser21) of insulin-treated samples are shown. Beta actin levels were analyzed and no changes were observed (blots not shown, refer to Figure 3.17). Results are presented as means ± SE (n = 4) * $P < 0.05$ versus control.

3.5.5 SAPK/JNK phosphorylation

We then analyzed the effect of BSO on JNK phosphorylation at Thr183/Tyr185. HepG2 cells were incubated with 3 mM of BSO for 48 hours, followed by 100 nM insulin incubation for 30 minutes. No significant difference in JNK phosphorylation was observed and p46 was not detectable. This indicates that the concentration of BSO selected was not causing extra stress after 48-hour incubation in serum-free media compared to control in which stress is present due to long incubation time in serum-free media. Results are shown in Figure 3.16.

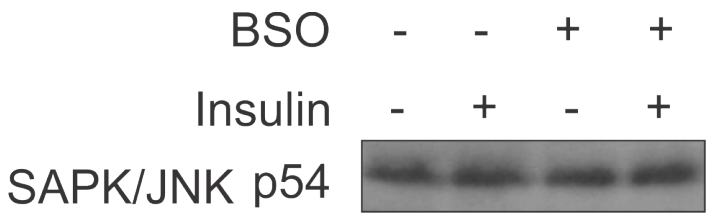

Figure 3.16 Effect of BSO on SAPK/JNK (Thr183/Tyr185) phosphorylation. SAPK/JNK phosphorylation following 48-hour incubation with 3 mM BSO followed by 30 minutes incubation with 100 nM insulin. The blot is representative of three independent experiments.

3.5.6 FoxO and Akt phosphorylation in the presence of glutathione

To investigate whether the BSO effect on insulin-induced FoxO phosphorylation was related to the specific depletion of glutathione caused by BSO, cells were incubated in the presence of membrane-permeable GEE was incubated after BSO treatment to restore or replenish intracellular glutathione levels. Insulin-induced Akt and FoxO phosphorylations were investigated. HepG2 cells were incubated with 3 mM BSO for 48 hours followed by 5 mM GEE for 3 hours followed by 100 nM insulin treatment for 30 minutes. Interestingly, GEE significantly reversed the effect of BSO on insulin-induced FoxO phosphorylation when the stock of GEE was prepared in PBS without adjusting the pH (pH = 6.3). However, after using a different GEE stock in a strong phosphate buffer with adjusted pH (pH = 7), the effect of BSO on insulin-induced FoxO phosphorylation was not reversed. This may be due to the fact that under slightly acidic conditions, GEE is more membrane-permeable in the protonated form, allowing it to enter the cells and restore GSH levels more effectively compared to the GEE stock in neutral pH condition. However, the pH in the treatment solutions obtained from both GEE stocks was similar (pH = 8.2) on the pH-indicating litmus paper. The following Figure 3.17 contains data obtained after using GEE stock with non-adjusted pH (pH = 6.3).

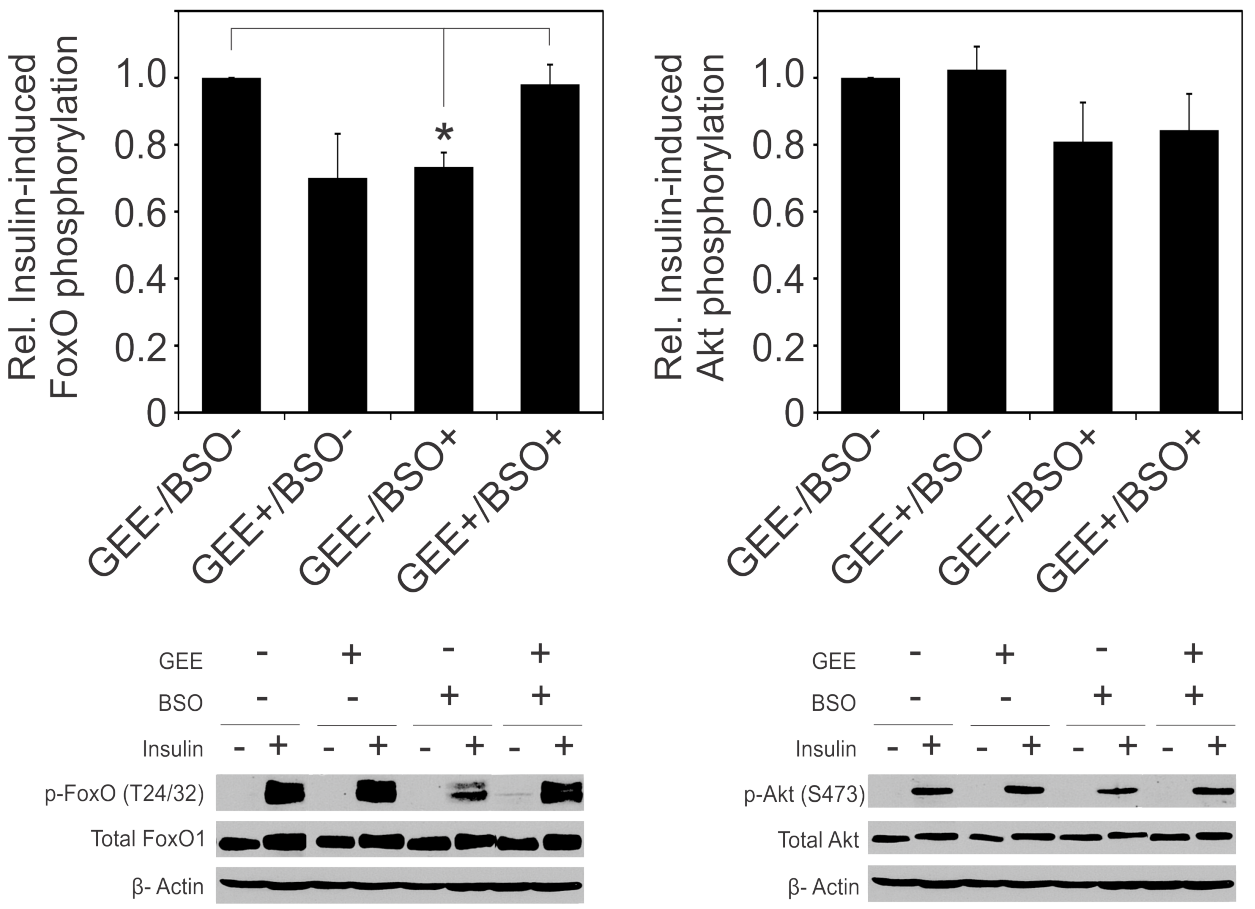

Figure 3.17 Effect of BSO on insulin-induced Akt (Ser473) and FoxO phosphorylation (Thr24/Thr32) in the presence of glutathione. Akt phosphorylation and FoxO phosphorylation following 48-hour incubation with 3 mM BSO followed by 3-hour incubation with 5 mM GEE followed by 100 nM insulin treatment for 30 min. For densitometric analysis, p-Akt signals were normalized against total Akt levels while p-FoxO signals were normalized against total FoxO1 levels. Beta actin levels were analyzed to test for alterations in total Akt and total FoxO1 levels. Results are presented as means ± SE (n = 5) * $P < 0.05$ versus control and GEE+/BSO+.

CHAPTER 4

DISCUSSION

4.1 General discussion

FoxO transcription factors are known mediators of insulin signaling (Manning and Cantley 2007). FoxO activity is tightly regulated by oxidative stress or cellular redox state (Ponugoti et al. 2012). GSH, the most abundant intracellular antioxidant, is directly involved in maintenance of cellular redox state. GSH/GSSG ratio has been used as an indicator of cellular oxidative stress for decades (Asensi et al. 1999). Therefore, GSH may play a role in regulating FoxO signaling and activity. In addition, the FoxO4 isoform has been reported to have a reactive cysteine residue (Dansen et al. 2009). Glutathiolation of reactive cysteine residues is an established regulatory process of several transcription factors (Pineda-Molina et al. 2001; Xie et al. 2009). Two chemical agents were selected in this work to modify GSH levels, DEM and BSO. DEM conjugates and depletes glutathione levels in the presence of glutathione-S-transferases, however it also depletes and conjugates other intracellular thiols due to its strong electrophilic nature (Adams et al. 1983; Bannai 1984; Aoshiba et al. 1999). DEM also has an effect on Crm1-mediated export of proteins (Crampton et al. 2009). In our study, DEM had a profound blocking effect on insulin-induced FoxO phosphorylation. Insulin-induced FoxO phosphorylation at Thr24 for FoxO1 and Thr3 for FoxO3a was significantly attenuated after DEM treatment. This effect was concentration-dependent. In addition, DEM treatment was associated with general impairment of insulin signaling which could partially explain the blocking effect on insulin-induced FoxO phosphorylation. DEM at high concentrations (10 mM) attenuated insulin receptor tyrosine phosphorylation, Akt phosphorylation (in a 2-hour incubation) and GSK3 alpha phosphorylation in the presence of insulin as a stimulant. However, DEM at lower concentrations (from 1 to 3 mM) for 2 hours induced insulin-stimulated Akt phosphorylation. This may be due

to prolonged generation of ROS which are known to activate Akt. To eliminate the possibility that the DEM effect on FoxO signaling is due to the known DEM effect on Crm1, a specific Crm1 inhibitor, LMB was used. Interestingly, LMB had no effect on insulin-induced FoxO phosphorylation i.e. it did not affect FoxO phosphorylation as with DEM. LMB also did not have any effect on insulin-induced Akt phosphorylation. Moreover, reversing the treatment protocol i.e. treating with insulin first followed by DEM, has also resulted in strong attenuation of insulin-induced FoxO phosphorylation. This indicates that DEM effect on FoxO phosphorylation is not solely dependent on DEM effect on Crm1/nuclear export if at all. DEM was reported to induce cellular phosphatases (Esposito et al. 2000). To further investigate the mechanism of DEM effect on FoxO signaling, co-incubation of phosphatase inhibitors with DEM was performed. Interestingly, presence of phosphatase inhibitors like OA and CA did not reverse DEM effect on insulin-induced FoxO phosphorylation, indicating that the blocking effect seen on insulin-induced FoxO phosphorylation is not due to phosphatases induction. On the other hand, stress-activated kinase JNK was strongly activated with DEM. In line with these findings, FoxO localization studies in our lab have shown that DEM induced FoxO nuclear accumulation (data not shown). Overall, DEM impairs insulin signaling in general and activates JNK pathway. Knowing that JNK activation results in c-Jun-dependent phosphorylation of FoxO and nuclear accumulation (Greer and Brunet 2005), we concluded that DEM-activated JNK pathway may be responsible for the effect of DEM on insulin-induced FoxO phosphorylation. JNK activation may be causing an alteration of FoxO response to insulin stimulation. To correlate glutathione/ thiol depletion with DEM effect on insulin-induced FoxO phosphorylation, we performed DEM co-incubation experiments to replenish glutathione/thiol levels. N-acetyl cysteine (NAC) or

glutathione ethyl ester (GEE) or reduced glutathione (GSH) were co-incubated with DEM followed by insulin treatment. We found that presence of these glutathione/thiol replenishing agents with DEM significantly reversed DEM effect on insulin-induced FoxO phosphorylation. However, knowing that GSH is not cell membrane-permeable and still reversed DEM effect on insulin-induced FoxO phosphorylation when co-incubated with DEM suggests that DEM interacts directly with GSH. To confirm that DEM interacts with GSH even without the presence of glutathione-S-transferases, DEM was mixed with GSH in test tubes and thiol determination was performed. Thiol levels were significantly less in the DEM-GSH samples compared to GSH samples alone. This indicates that reversing DEM effect on insulin-induced FoxO phosphorylation following co-incubation with NAC, GEE and GSH was simply due to direct interaction with DEM, lowering the latter effect. Summary of DEM effects is provided in Figure 4.1.

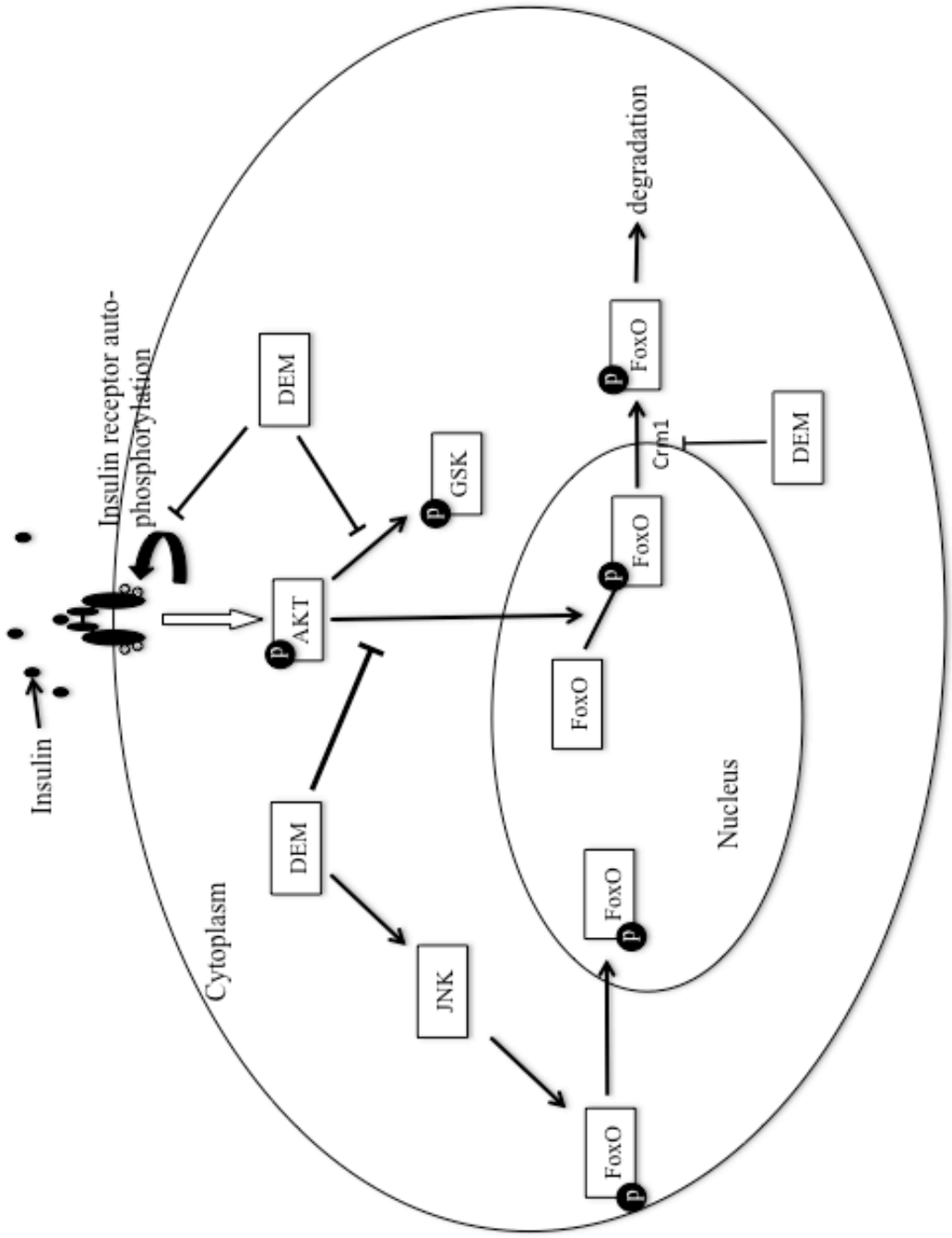

Figure 4.1 Summary of DEM effects on FoxO signaling

BSO is a specific inhibitor of gamma glutamyl synthetase, an enzyme required for the first step in the synthesis of glutathione, was shown to significantly attenuate insulin-induced FoxO phosphorylation. Insulin-induced Akt phsophorylation was also significantly attenuated. As for insulin-induced insulin receptor tyrosine phosphorylation and GSK alpha phosphorylation, a non-significant trend of attenuation was observed. JNK activation was observed equally in BSO-treated and control; this is due to the long 48-hour incubation in serum free media i.e. BSO did not have an effect on JNK activation. These findings indicate that specific glutathione depletion by BSO significantly impaired insulin-induced FoxO phosphorylation. In addition, restoring GSH levels after 48-hour incubation with BSO by using GEE stock (pH 6.3) has reversed BSO effect on insulin-induced FoxO phosphorylation i.e. insulin-induced FoxO phosphorylation was restored. Interestingly, using GEE stock (pH 7) did not have the same effect as with using GEE stock (pH 6.3) i.e. insulin-induced FoxO phosphorylation was not restored. This may be because in slightly acidic conditions, GEE is more membrane-permeable allowing it to restore GSH levels more effectively.

Several findings can be summarized from this work. First of all, DEM interferes with FoxO signaling in multiple possible ways. DEM blocks insulin-induced FoxO phosphorylation at Thr24 for FoxO1 and Thr32 for FoxO3a. Knowing that insulin-induced FoxO phosphorylation is caused by activated Akt, the mechanism behind this effect may be attributed to the following: DEM attenuates insulin-induced insulin receptor tyrosine phosphorylation at Tyr1150/1151, GSK-3 alpha phosphorylation at Ser21, and at higher levels and prolonged incubation attenuates insulin-induced Akt phosphorylation at Ser473. In addition, DEM also activates JNK stress

pathway which is known to affect FoxO signaling and activity. The simple activation of JNK may be responsible for altering FoxO susceptibility to insulin-induced phosphorylation. Another possibility of DEM effect on insulin-induced FoxO phosphorylation is the interaction of DEM with cysteine residues directly nearby Thr24 in FoxO1 and Thr32 in FoxO3a as demonstrated in Figure 4.2. This interaction may be causing structural hinderance preventing or repelling Akt from phosphorylating FoxO. DEM effect on nuclear export/import machinery seems to play little or no role in DEM's effect on insulin-induced FoxO phosphorylation. The exact mechanism of DEM effect on insulin-induced FoxO phosphorylation remains to be investigated further. Second of all, BSO interferes with FoxO signaling as well. Specific depletion of glutathione attenuated insulin-induced FoxO phosphorylation at Thr24 for FoxO1 and Thr32 for FoxO3a. Insulin-induced Akt phosphorylation at Ser473 was significantly attenuated as well. Phosphorylation of other downstream targets in insulin signaling were slightly attenuated to some extent, including insulin-receptor tyrosine phosphorylation at Tyr1150/1151 and GSK-3 alpha phosphorylation at Ser21. Following depletion of glutathione and then restoration of glutathione, insulin-induced FoxO phosphorylation was restored. This indicates that glutathione is indeed involved in insulin downstream signaling on FoxO.

As for other post-translational modifications of FoxO, they were not part of the objective of this work. However, knowing that acetylation is influenced by oxidative stress, it would be of interest to check the acetylation status of FoxO after exposure to glutathione-depleting agents and correlating the findings with the observed effect on insulin-induced FoxO phosphorylation if plausible. On the other hand, investigating the effect of glutathione-depleting agents on FoxO

interaction with known binding partners may also be of interest. However, this was outside the scope of our research objective. The clinical relevance of this work lies in correlating various disease states pertaining to FoxO to our findings, which requires in vivo animal observational studies. Performing these studies may result in discovery of novel strategies involving a role for glutathione in the treatment of FoxO-related diseases such as cancer and diabetes

4.2 Conclusion

Knowing that FoxO transcription factors mediate insulin effects and activates cell cycle arrest pathways, researchers have viewed FoxOs as potential targets for type II diabetes and cancer therapy (Maiese et al. 2008; Lu and Huang 2011). However, finding optimal strategies to modulate FoxO protein levels is a difficult task. This is due to the ability of FoxO to regulate several important physiological functions at the same time, such as In this work, we have identified a novel method to regulate FoxO response to insulin signaling. This method involves modulation of glutathione levels. Glutathione has been known for its role in insulin signaling (Najib and Sanchez-Margalet 2001). However, previous literature reports discussing the correlation between glutathione and FoxO transcription factors is lacking. We have shown for the first time that depletion of glutathione alone resulted in impairment of insulin-induced FoxO phosphorylation. This provides additional evidence for the importance of glutathione as a potential marker in insulin signaling. Finally, the findings in this work could be of clinical importance for type II diabetes and future development of FoxO modulators.

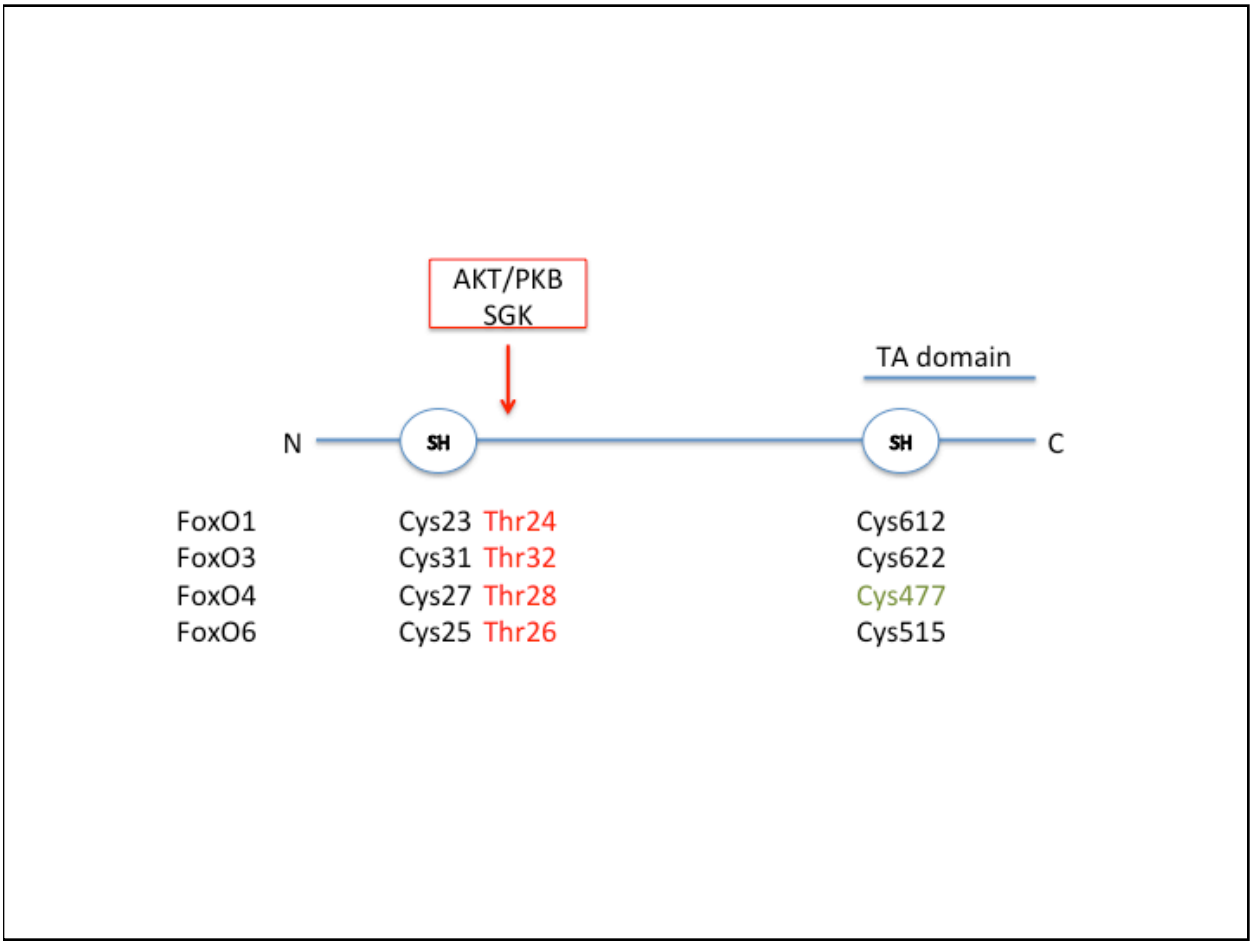

Figure 4.2 Conserved cysteines in FoxO structure. FoxO isoforms contain two conserved cysteine residues, one located immediately before the Akt-responsive threonine sites in the N-terminal, the other located in the transactivation (TA) domain at the C-terminal. These cysteines may play a role in FoxO regulation. Green-colored cysteine has already been reported in the literature to be reactive under oxidative stress conditions (Dansen et al. 2009).

4.3 Future directions

Several other agents can modulate GSH levels aside from DEM and BSO. Menadione and diamide are known modulators of GSH/GSSG levels (Kosower and Kosower 1995; Abdelmohsen et al. 2004). Investigation of FoxO signaling after depleting glutathione with menadione and diamide could be of interest. Further studies are required to understand the mechanism of FoxO regulation by glutathione and can be summarized as follows:

1. Investigation of direct FoxO glutathionylation after modulation of glutathione levels
2. Investigation of other FoxO phosphorylation sites known for their role in governing FoxO cytoplasmic or nuclear accumulation
3. Use of JNK inhibitors with DEM treatment to further confirm that JNK activation by DEM blocked/reversed insulin-induced FoxO phosphorylation resulting in nuclear accumulation
4. Use of animal models to further confirm the regulatory role of glutathione on FoxO signaling in vivo
5. Investigation of other FoxO sites phosphorylated by insulin after DEM treatment

CHAPTER 5

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