O-GLCNAC TRANSFERASE MODULATES JNK1 AND FOXO4 TRANSCRIPTION FACTOR TO RESIST ACUTE OXIDATIVE STRESS

by

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O-GlcNAc Transferase Modulates JNK1 and FOXO4 Transcription Factor to Resist Acute Oxidative Stress

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Cell Biology

Abstract

O-GlcNAcylation is an abundant and dynamic post-translational modification on serine and threonine residues of nuclear and cytoplasmic proteins. O-GlcNAc Transferase (OGT) and Nuclear Cytoplasmic O-GlcNAcase and Acetyltransferase (NCOAT) are the only two enzymes and major regulators in this process. In the past two decades, many studies have demonstrated its important modulation roles in nutrient sensing, protein turnover, cell cycle progression, transcription, translation as well as stress resistance. Among these biological functions, the understanding of O-GlcNAcylation dependent oxidative stress is relatively unknown. This study aims to examine the effects of O-GlcNAcylation on JNK1 and FOXO4 transcription factors under acute oxidative stress treatment.

Oxidative stress is an imbalance between production and clearance of reactive oxygen species (ROS) in cells. Overload oxidative stress may cause many aging-related diseases including Type II diabetes, neurodegenerative disorders and muscle atrophy. MAPK death signal and PI3 kinase survival signal are two of the major evolutionary conserved phosphorylation cascade pathways involved in oxidative stress mediated cellular response. In this study, we show that OGT overexpression in cells have a high survival rate and a low cell death under acute hydrogen peroxide. Further studies found that OGT specifically associates with, and O-GlcNAcylates, JNK1 but not ERK1 and p38-MAPK. Oxidative stress induced JNK1 O-GlcNAcylation also prevented
phosphorylation-dependent death signal transduction. On the other hand, hydrogen peroxide induced OGT interaction with FOXO4 and enhanced its transcriptional activity on survival target gene regulation.

Overall, the current work demonstrates OGT and O-GlcNAcylation can positively regulate the FOXO4 survival signal, and can negatively modulate the JNK1 death signal to protect cells from acute oxidative stress. These data provide one molecular mechanism to explain O-GlcNAcylation-dependent stress resistance and cell survival upon acute oxidative stress as well as other stress stimuli.

Keywords: O-GlcNAc Transferase, Oxidative stress, JNK1, FOXO4
DEDICATION

I dedicate the enclosed work to my parents I-Lan Ho and Meng-Jin Yang, and my mentor, Drs. Jeffrey E. Kudlow and Andrew J. Paterson for their support, understanding and patience.
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## SUMMARY

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### LIST OF ABBREVIATIONS

- **6xDBE**: 6 repeats of FOXO responsive DNA binding element
- **A**: adenine
- **A.A.**: amino acid
- **Ab**: antibody
- **Ac**: acetyl
- **Ag**: antigen
- **AKT**: one of serine/threonine-specific protein kinases/ PKB
- **ALX**: Alloxan
- **AMP**: adenosine monophosphate
- **Arg**: arginine
- **Asn**: asparagine
- **Asp**: aspartate
- **ATP**: adenosine triphosphate
- **BSA**: bovine serum albumin
- **c-fos**: a cellular proto-oncogene
- **c-jun**: c-fos-binding protein p39
- **C-terminal**: carboxy-terminal
- **C. elegans**: Caenorhabditis elegans
- **cAMP**: cyclic-AMP
LIST OF ABBREVIATIONS (Continued)

CNS  central nervous system
Co-IP  coimmunoprecipitation
COOH  Carboxyl
CTD  C-terminal domain
Cys  cysteine
DNA  deoxyribonucleic acid
E. coli  Escherichia coli
E2  estrodiol
EGFP  enhanced green fluorescent protein
ER  estrogen receptor
ERK  extracellular signal-regulated kinase
ETD  Electron Transfer Dissociation
FOXO  Forkhead box O
Fruct-6-P  fructose-6-phosphate
GDP  guanosine diphosphate
GFAT  glutamine: fructose-6-phosphate amidotransferase
GK  Goto-Kakizaki
Glc  glucose
Glc-6-P  glucose-6-phosphate
GlcN-6-P  glucosamine-6-phosphate
GlcNAc  N-acetylglucosamine
Gln  glutamine
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<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphate exchange factor</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H4</td>
<td>histone core particle 4</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
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<td>HBP</td>
<td>Hexosamine Biosynthesis Pathway</td>
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<td>HEK 293 cells</td>
<td>Human Embryonic Kidney 293 cells</td>
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<td>HepG2</td>
<td>a human liver carcinoma cell line</td>
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<td>HSP</td>
<td>Hexosamine Signaling Pathway</td>
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<td>IGFR</td>
<td>Insulin-like Growth Factor Receptor</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>InsR</td>
<td>insulin receptor</td>
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<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
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<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
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<td>JNK1</td>
<td>c-Jun N-terminal protein kinase 1</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>km</td>
<td>enzyme kinetics unit</td>
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<tr>
<td>kᵢ</td>
<td>Competitive Inhibition unit</td>
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<tr>
<td>LSB</td>
<td>low stringency buffer</td>
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<td>Lys</td>
<td>lysis</td>
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LIST OF ABBREVIATIONS (Continued)

mAb  monoclonal antibody
MAP  mitogen-activated protein
MAPK  mitogen-activated protein kinase
MAPKK  mitogen-activated protein kinase kinase
MAPKKK  mitogen-activated protein kinase kinase kinase
Mdm2  murine double minute oncogene protein
MEK-1  a dual threonine and tyrosine recognition kinase
MGEA5  meningioma expressed antigen 5
MKK3/6  Mitogen-Activated Protein Kinase Kinase 3/6
MKK4/7  Mitogen-Activated Protein Kinase Kinase 4/7
MnSOD  manganese superoxide dismutase
mOGT  mitochondrial OGT
mRNA  messenger ribonucleic acid
MS  mass spectrometry
mSin3A/B  mammalian homolog of yeast repressor Sin3A or B
N-terminal  amino terminal
NaCl  sodium chloride
NCOAT  nuclear and cytoplasmic O-GlcNAcase and acetyl transferase
ncOGT  nuclear and cytoplasmic OGT
NH4  ammonium
NLS  nuclear localization sequence
O-GlcNAc  O-linked N-acetylg glucosamine
LIST OF ABBREVIATIONS (Continued)

O-GlcNAcase  β-O-linked N-acetylglucosaminidase
OGN        O-GlcNAcase
OGT        O-GlcNAc transferase
p38-MAPK   p38 mitogen-activated protein kinase
PARP-1     Poly [ADP-ribose] polymerase 1
PBS        phosphate-buffered saline
PDK1       Phosphoinositide-dependent protein kinase 1
PI3        phosphoinositide-3
PI3 kinase phosphoinositide-3 kinase
PIP2       phosphoinositol 4,5 bisphosphate
PIP3       phosphotidylinositol-3,4,5-triphosphate
PKB        protein kinase B
PKC        protein kinase C
PLCβ       phospholipase C-beta
PtdIns     Phosphatidylinositol
PtdIns3Ps  phosphatidylinositol 3-phosphates
PTEN       phosphatase and tensin homolog
PTM        post-translational modification
PUGNAc     O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate
Ras        a transforming oncogene protein
RLU        raw light units
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<td>RNA</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>S.E.</td>
<td>standard error</td>
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<tr>
<td>SD</td>
<td>Sprague Dawley</td>
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<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEC</td>
<td>SECRET AGENT</td>
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<tr>
<td>Ser</td>
<td>serine</td>
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<td>SIRT1</td>
<td>Sirtuin 1</td>
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<td>sOGT</td>
<td>small OGT</td>
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<tr>
<td>Sp1</td>
<td>SV40 promoter activator 1</td>
</tr>
<tr>
<td>SPY</td>
<td>SPINDLY</td>
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<tr>
<td>STZ</td>
<td>streptoztocin</td>
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<tr>
<td>TGFα</td>
<td>Transforming growth factor alpha</td>
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<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>TPR</td>
<td>tetratricopeptide repeat</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
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<tr>
<td>UDP</td>
<td>uracil-diphosphate</td>
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<tr>
<td>UDP-GlcNAc</td>
<td>UDP-N-acetylglucosamine</td>
</tr>
<tr>
<td>UMP</td>
<td>uracil-monophosphate</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>WCL</td>
<td>whole cell lysate</td>
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LIST OF ABBREVIATIONS (Continued)

| WGA       | wheat germ agglutinin |
INTRODUCTION

Our dependence on nutrition may result in stress. The ability to adapt to nutrition flux is critical to cell survival, proliferation and death, from yeast to mammals (Holzenberger et al., 2000). As the primary form of nutrient in most living species, glucose provides ATP and modulates cell growth and metabolism (McClain et al., 1992). Generally, multiple cell organisms have a precise and complex control system to maintain glucose balance. Interruption of this balance in either overload or deprivation, causes cell and tissue damage and hence diseases (Olson and Pessin, 1996). Therefore, the regulation of glucose metabolism is involved in many conserved pathways. Hyper-glycemia mediated oxidative stress induced PI3 kinase and MAPK signaling is thought to be one of the major pathophysiological factors causing late complications in diabetes (Evans et al., 2002; Brownlee, 2005). In addition, emerging evidence points out the hexosamine biosynthesis pathway (HBP) may serve as a nutrient sensor for the regulation of glucose uptake, glycogen synthesis, glycolysis, and the synthesis of growth factors as well as play a part in stress response (McClain et al., 1992; Yang et al., 2008). HBP may play an important role in stress resistance and cell survival.

Hexosamine Biosynthesis Pathway (HBP) and Protein O-GlcNAcylation

The HBP is a relatively minor branch of glycolysis. Upon glucose entering into the cell through specific glucose transporters, approximately 2-5% glucose will be
**Figure 1. Hexosamine Biosynthesis Pathway and protein O-GlcNAcylation.** Glucose entry into cells through specific glucose transports (Glut), then Hexokinase first phosphorylates glucose on the C6 hydroxyl group and then converts it to glucose-6-phosphate (Glc-6-P). Glc-6-P is further converted to fructose-6-phosphate (Fruct-6-P) by phosphoglucone isomerase. In the next step, approximately 2-5% of fructose-6-phosphate is converted to glucosamine 6-phosphate, catalyzed by the first and rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) of HBP. The major end product of HBP is UDP-N-acetylglucosamine (UDP-GlcNAc), which is served as substrate for protein O-GlcNAcylation process by OGT and NCOAT proteins.
utilized via HBP. Hexokinase first phosphorylates glucose on the C6 hydroxyl group and then converts it to glucose-6-phosphate (Glc-6-P). Glc-6-P is further converted to fructose-6-phosphate by phosphoglucose isomerase. At this step, fructose-6-phosphate is converted to glucosamine 6-phosphate, catalyzed by the first and rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) of HBP. The major end product of HBP is UDP-N-acetylglucosamine (UDP-GlcNAc) which is served as substrate for protein O-GlcNAcylation process (Figure. 1)(Buse, 2006).

Protein O-GlcNAcylation is a dynamic cycle of addition and removal of O-GlcNAc at serine and threonine residues of nuclear and cytoplasmic naked proteins. Since discovered in 1984(Torres and Hart, 1984), it has emerged as a key regulator of nuclear and cytoplasmic proteins activity [see reviews in (Zachara and Hart, 2006; Hart et al., 2007; Butkinaree et al., 2009; Hanover et al., 2009; Macauley and Vocadlo, 2009b)]. Like phosphorylation, protein O-GlcNAcylation dramatically alters the posttranslational fate and function of target proteins and may compete with phosphorylation for certain Ser/Thr target sites(Kudlow, 2002; Zachara and Hart, 2002, 2004; Zachara et al., 2004; Kudlow, 2006; Zachara and Hart, 2006). Similar to kinases and phosphatases, the enzymes of O-GlcNAc metabolism are highly compartmentalized and regulated. Yet, O-GlcNAc addition is subject to an additional and unique level of metabolic control. O-GlcNAc addition is the terminal step in a “hexosamine signaling pathway” (HSP). In the HSP, levels of uridine 5'-diphosphate (UDP)-GlcNAc respond to nutrient excess to activate O-GlcNAcylation. Removal of O-GlcNAc may also be under similar metabolic regulation. O-GlcNAc addition and removal is critical to histone remodeling, transcription, proliferation, apoptosis, and proteasomal degradation. This nutrient-
responsive signaling pathway also modulates important cellular pathways including the insulin signaling cascade in animals and the gibberellin signaling pathway in plants. Alterations in O-GlcNAc metabolism are associated with various human aging-related diseases including diabetes mellitus and neurodegeneration (Love and Hanover, 2005).

**O-GlcNAc Transferase (OGT)**

O-GlcNAc modification has been identified in all multicellular eukaryotes and consistent with this, homologs of OGT have been identified in *Caenorhabditis elegans (C. elegans)*, *Drosophila* and *Arabidopsis*, but not in *Saccharomyces cerevisiae* (Kreppel et al., 1997; Lubas et al., 1997; Hartweck et al., 2002; Gambetta et al., 2009). Human OGT has 99% identity with rat OGT (Kreppel et al., 1997). In mammalian cells, OGT is encoded by a single copy X-linked gene (Kreppel et al., 1997; Lubas et al., 1997; Shafi et al., 2000), while in other species, there are two homologs spy and secret agent genes in plants (Hartweck et al., 2002) and the two homologs ogt copy I and copy II genes in zebra fish (Webster et al., 2009). Therefore, knockout ogt gene causes development defects and even embryonic lethality in mice (Shafi et al., 2000) but not in zebra fish (Webster et al., 2009) and worms (Hanover et al., 2005). The development defect may be explained by recent studies that show OGT serving as an essential polycomb repression regulator in fly development (Gambetta et al., 2009; Sinclair et al., 2009). OGT is expressed in all tissues studied, although it appears to be particularly rich in the pancreas, brain, and thymus (Kreppel et al., 1997; Lubas et al., 1997).

OGT contains three parts - an N-terminal tetraticopeptide repeat (TPR) domain, a bipartite nuclear localization sequence (NLS) and a C-terminal catalytic domain (Kreppel
et al., 1997; Lubas et al., 1997) - and is located predominantly within the nucleus as determined by immunofluorescence and subcellular fractionation. In mammalian cells, splicing of OGT mRNA leads to at least three alternative transcripts: mitochondrial OGT (mOGT), nucleocytoplasmic OGT (ncOGT) and small OGT (sOGT)(Kreppel et al., 1997; Lubas et al., 1997; Nolte and Muller, 2002). The 78-kDa sOGT has 2 TPR repeats and localizes in both nucleus and cytoplasm of some tissues such as muscle. The 103-kDa mOGT has 9 TPR repeats and an alternative mitochondrial targeting sequence N-terminus that causes it only to exist in the mitochondria(Hanover et al., 2003; Love et al., 2003). The function of sOGT and mOGT is less characterized.

The 110-kDa ncOGT, encoded by 1037-amino-acid protein, was thought to exist predominantly either as a homotrimer comprised of identical 110-kDa subunits or as a heterotrimer comprised of 2 identical 110-kDa subunits coupled with a 78-kDa subunit(Kreppel et al., 1997; Lubas et al., 1997; Akimoto et al., 1999). The OGT is potently inhibited by NaCl, UDP, UMP, the uracil analog alloxan, and N-ethylmaleimide(Haltiwanger et al., 1992; Konrad et al., 2002; Okuyama and Marshall, 2003; Gross et al., 2005; Lazarus et al., 2005; Hurtado-Guerrero et al., 2008; Pathak et al., 2008). Mutational studies have highlighted key residues in the C-terminus, especially Cys residue C839, that contribute to OGT activity. Replacement of C839 with a Ser residue results in a 93% reduction in activity(Lazarus et al., 2005).

The rat ncOGT has 11.5 TPR repeats which are important for inter-subunit interactions, substrate recognition and protein–protein interactions(Kreppel and Hart, 1999; Lubas and Hanover, 2000; Beck et al., 2002; Yang et al., 2002; Iyer et al., 2003; Iyer and Hart, 2003a, b; Jinek et al., 2004). Kreppel and co-workers have shown that
deletion of TPR 6–11.5 resulted in monomeric OGT (Kreppel and Hart, 1999). Deletion of the first three TPR domains does not affect activity of ncOGT toward peptides, but it ablates activity toward full-length CK2 and nuclear pore protein p62 (np62) (Kreppel and Hart, 1999; Lubas and Hanover, 2000). The affinity of OGT for the full-length protein np62 is higher than for peptides (Shafi et al., 2000). Collectively, these data suggest a role for the TPR domain in mediating substrate specificity and binding. The role of the TPR domain in mediating substrate recognition may be one reason why a consensus motif for the addition of O-GlcNAc has not been identified. Other studies also demonstrate OGT can interact with phosphorylated p38-MAPK (Cheung and Hart, 2008) and phosphoinositides (Yang et al., 2008) through the C-terminal area. The mechanism(s) by which “one” OGT specifically modifies myriad proteins are unclear, although it appears that UDP-GlcNAc concentration, protein–protein interactions, glycosylation, and phosphorylation may all play a role. Several proteins have been identified that interact with OGT, and while these interactions are not known to change the activity of OGT, they do act to anchor and/or target OGT to signaling and transcriptional complexes (Beck et al., 2002; Yang et al., 2002; Iyer et al., 2003; Iyer and Hart, 2003a, b). These interactions may modulate the activity of OGT by affecting its localization, modulating the binding of substrate proteins, or targeting to complexes where it is specifically activated by signal transduction events. Notably, OGT is both O-GlcNAc modified and tyrosine phosphorylated (Kreppel et al., 1997; Lubas and Hanover, 2000; Dephoure et al., 2008; Yang et al., 2008). Finally, the K_m of OGT for various peptide substrates appears to change at different UDP-GlcNAc concentrations, suggesting that as levels of UDP-GlcNAc change within the cell, OGT will target a different population of
substrates (Kreppel and Hart, 1999). The coordinate regulation of substrate binding, substrate preference, association with target complexes, and post-translational modification may mediate OGT in such a way that it specifically modifies different proteins in response to diverse signals, appropriately regulating cellular function.

**Nuclear Cytoplasmic O-GlcNAcase and Acetyltransferase (NCOAT)**

NCOAT (also known as O-GlcNAcase, mgea5) is a soluble, cytosolic β-N-acetylglucosaminidase expressed in all tissues examined and predominantly in immune cells and the brain. It is well conserved in mammals, with 97.8% identity between the human and mouse gene, and 29% identity (and 43% homology) between the human and *C. elegans* gene (Gao et al., 2001; Wells et al., 2002). The 130-kDa NCOAT is encoded by 917-amino-acid protein and has two functional domains, a N-terminal hexosaminidase domain and a C-terminal histone acetyltransferase (HAT) domain (Toleman et al., 2004). The NCOAT sequence was originally identified as a hyaluronidase auto-antigen in meningioma and named as MGEA5 and may have some activity against hyaluronan (Heckel et al., 1998; Comtesse et al., 2001; Gao et al., 2001; Farook et al., 2002; Toleman et al., 2004).

Two natural splicing variants of NCOAT, in which amino acids 250–345 (exon 8) and 250–398 (exons 8 and 9) are deleted, have no O-GlcNAcase activity suggesting that the O-GlcNAcase active site is within the N-terminal half of this protein (Toleman et al., 2004). Interestingly, another splice variant is coded by the first 10 exons and part of intron 10, resulting in a 677-amino-acid nuclear protein that has little or no O-GlcNAcase activity (Comtesse et al., 2001; Macauley and Vocadlo, 2009a). The physiological
relevance of these variants has not been well defined, although the Δ250–345 NCOAT is found in Goto-Kakizaki Wistar (GK) rats, at a locus associated with a high frequency of type II diabetes(Farook et al., 2002; Kudlow, 2002).

The C-terminal domain of O-GlcNAcase has homology to HATs at residues AA 772–899 and was recently shown to acetylate both free core histones and nucleosomal histone proteins(Heckel et al., 1998; Comtesse et al., 2001; Gao et al., 2001; Farook et al., 2002; Toleman et al., 2004). Mutation of key aspartic acid (D853N and D884N) and phenylalanine residues (F891Y) ablates activity. Consistent with these data, the HAT activity was mapped to amino acid residues 583–917 of the protein(Toleman et al., 2004). OGT is known to associate with histone deacetylase complexes and to promote transcriptional silencing(Yang et al., 2002). Together, these data suggest that dynamic modification of transcriptional complexes by O-GlcNAc and histone acetylation play key roles in transcriptional regulation. It has been shown that NCOAT is cleaved at Asp413 residue by Caspase-3(Butkinaree et al., 2008). However, this does not appear to modify the activity of either the O-GlcNAcase or the HAT domains(Wells et al., 2002; Toleman et al., 2004; Butkinaree et al., 2008). The role of the cleavage is unknown, although it may deregulate O-GlcNAcase during programmed cell death.

NCOAT co-purifies with a complex of proteins suggesting that, like OGT, it is regulated by its interactions with other proteins(Gao et al., 2001; Wells et al., 2002). Moreover, NCOAT can interact with OGT TPR domains 1-6 to form the O-GlcNAc complex and regulate proper estrogen-dependent cell signaling as well as mammary development in transgenic mice(Whisenhunt et al., 2006). In addition, it has been shown that NCOAT is Ser364, Thr709 phosphorylated(Beausoleil et al., 2004; Dephoure et al.,
2008; Tsai et al., 2008) and Ser405 O-GlcNAcylated (Khidekel et al., 2007), suggesting an additional mechanism of regulation. O-GlcNAcase activity is efficiently inhibited by O-(2-acetamido-2-deoxy-d-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAC; \( K_i \ 54 \text{ nm} \))(Dong and Hart, 1994; Haltiwanger et al., 1998) and Streptozocin (STZ), and recent studies have isolated two new inhibitors of O-GlcNAcase (NAG-thiazoline and NButGT) which may be more specific(Macauley et al., 2005; Macauley and Vocadlo, 2009b).

**O-GlcNAc metabolism, oxidative stress and aging-related diseases**

The enzymes of O-GlcNAc cycling are involved in many aging-related diseases. OGT is encoded on chromosome Xq13, which has a high susceptibility for X-linked Dystonia Parkinsonism(Muller et al., 1994; Haberhausen et al., 1995). Furthermore, a large-scale genetic association analysis reveals that NCOAT gene locus is highly relative to type-2 diabetes in the Mexican-American population(Lehman et al., 2005). Numerous evidence also point out O-GlcNAc cycling associates with cardiovascular disease, cancer, immune dysfunction, type-2 diabetes mellitus and neurodegeneration(Cole and Hart, 2001; Hanover, 2001). Our laboratory also demonstrates that tissue-specific overexpression of NCOAT dominant negative causes cataracts and muscle atrophy(Wang et al., 2009a).

Aging-related diseases are becoming more prevalent in modern society due to increasing numbers of older people. Generally, the normal aging process is characterized by a decrease in adaptive abilities due to a progressive failure of maintenance by some stress response proteins(Sonneborn, 2005). Stress response is defined as a signal
generated by any physical, chemical, or biological factor (stressor), which, in a living system, initiates a series of events in order to counteract, adapt, and survive. These stressors increase the formation of ROS to a level that may induce significant, but tolerable, damage that can in turn induce beneficial adaptations in keeping with anti-aging process (Rattan, 2004). Cells resist oxidative stress through a variety of complementary mechanisms including enzymatic and non-enzymatic detoxification, the production of protein chaperones, systems for the removal of damaged molecules and DNA repair enzymes. Most of the mechanisms cited above are enhanced when cells are subjected to mild stressors including oxidative, thermal and metabolic stress. This adaptive process causes cells (and organisms) to become resistant to increasing severe stress (Arumugam et al., 2006). For example, caloric restriction and exercise increase the resistance of organisms to stress, and activate stress resistance pathways in cells in different tissues throughout the body, and extend lifespan (Grossman, 2005). Although mild stress response influences are clearly encountered at the cellular and molecular level of aging process, little is known about the mechanisms.

**CONSERVED SIGNALINGS**

Forkhead transcription factors, FOXOs, seem to exist from yeast to humans. They confer tolerance to many kinds of stresses (particularly oxidative) and extend life span by inducing longevity genes. In humans, FOXO1, FOXO3a and FOXO4, as well as their downstream effectors, could hold the key to counteracting aging and common diseases. FOXO transcription factors appear to be the main effectors of protein kinase B (PKB)/AKT, which belongs to the evolutionarily conserved PI3 kinase signaling.
PKB/AKT promotes FOXOs export from the nucleus, increases the death rate, down-regulates antioxidant enzymes and heat shock proteins, and reduces the accumulation of fat or glycogen. In contrast to PI3 kinase signaling, another conserved MAPK signaling triggers c-jun and c-fos to activate stress response and apoptosis proteins. Oxidative stress and other stress stimuli are able to trigger both PI3 kinase signaling and MAPK signaling to decide cell survival or death. Both conserved pathways are modulated by many posttranslational modifications (PTMs). Phosphorylation is one of the well known PTMs among these oxidative stress mediated conserved signalings. On the other hand, recent studies also show that O-GlcNAcylation is involved in these conserved signaling and stress resistance responses (Cheung and Hart, 2008; Housley et al., 2008; Kuo et al., 2008; Housley et al., 2009). However, the detail mechanisms by which this happen still are not fully understood.

**PI3 kinase signaling pathway**

PI3 kinase signaling can be activated by many tyrosine kinase receptor pathways including the insulin/IGF-I receptor (InsR/IGFR) signaling pathways (Fig. 2) (Saltiel and Kahn, 2001). The activated InsR/IGFR phosphorylates and actives insulin receptor substrate 1 (IRS-1). Phosphorylated IRS-1 provides binding sites for the PI3 kinase heterodimer p85–p110 and results in the activation of PI3 kinase, following a conversion of phosphatidylinositol (PtdIns) lipids into 3’-phosphorylated phosphatidylinositol lipids (PtdIns3Ps). In contrast with PI3 kinase, the product of the tumor suppressor gene, *PTEN*, acts as a 3’-phosphatase and has been shown to lower the amount of PtdIns3P and
Figure 2. An insulin/insulin-like growth factor (IGF) signaling in mammals.

Activation of the insulin/IGF-I receptor (InsR/IGFR) initiates phosphorylation cascade signaling. InsR/IGFR activates PI3 kinase following induction of binding of the phosphatidylinositol-3-kinase (PI3 kinase) heterodimer p85–p110. Activated PI3 kinase converts phosphatidylinositol (PtdIns) lipids into 3’-phosphorylated phosphatidylinositol lipids (PtdIns3Ps) causing phosphoinositide kinase 1 (PDK1) and protein kinase B (PKB) to the plasma membrane, where PDK1 phosphorylates PKB at Thr308, activating PKB/AKT. Activated PKB/AKT phosphorylates the FOXO transcription factors to prevent its nuclear translocation(Burgering and Kops, 2002).
switch off PI3 kinase signaling in cell cultures (Fruman et al., 1998; Cantley and Neel, 1999). PtdIns3P recruits phosphoinositide kinase 1 (PDK1) and PKB/AKT to the plasma membrane, where PDK1 phosphorylates PKB at Thr308, activating PKB/AKT. Active PKB/AKT phosphorylates the FOXO transcription factors. In growth-factor signaling in general, and insulin signaling in particular, and bifurcation of signals is often observed. Two possibilities for bifurcation are indicated here. First, the activated InsR/IGFR can phosphorylate the adaptor protein Shc as well as IRS-1; through GTPase Ras. Second, the phosphorylated IRS-1 will recruit not only the p85–p110 PI3K complex but also other signaling molecules such as Fyn and NCK (Burgering and Kops, 2002).

The PI3 kinase can control key functions of the cell such as proliferation, apoptosis as well as cell aging. Experiments with *C. elegans* reveal a homology between aging gene Age1 and the PI3 kinase catalytic subunit in mammals and demonstrate the involvement of Age1 in the control of development of *C. elegans* (Morris et al., 1996). Convincing evidence for the involvement of PI3 kinase in the aging cell has been obtained from the experiments on normal fibroblasts by comparative analysis of the effects of PI3 kinase inhibitor LY294002 and MEK-1 (kinase phosphorylating extracellular signal-regulated protein kinase 1 (ERK1)/ERK2) inhibitor PD58029 in vitro (Tresini et al., 1998). This showed that the PI3 kinase-dependent control of the anti-apoptotic signaling pathway and cell is independent of the RAS/RAF/ERK cascade. In addition, data suggests that cyclic stretch activates the PI3 kinase signaling including PI3 kinase, AKT, FOXO1, and FOXO4. Moreover, it also causes the phosphorylation of JNK, and the depletion of calcium from the medium resulting in attenuation in the level of phosphorylation of these proteins (Danciu et al., 2003). In summary, PI3 kinase
signaling, in particular the FOXO transcription factor, modulates oxidative stress response and normal aging process.

**Mitogen-activated proteins kinase (MAPK) signaling pathway**

Four major MAPKs, the ERK1/ERK2, JNK, ERK5 and p38-MAPK, are evolutionarily highly conserved enzymes connecting cell-surface receptors to critical regulatory targets cascades within cells. MAPK cascades consist of a module of three cytoplasmic kinases: a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK), an MAP kinase kinase (MAPKK), and the MAP kinase (MAPK) itself (Figure 3)(Yoon et al., 2002). The MAPKKK is a serine-threonine kinase that receives activating signals from a membrane-spanning receptor and then phosphorylates and activates its substrate, an MAPKK. This enzyme is a dual-specificity kinase with the potential to phosphorylate critical threonine and tyrosine residues in its substrate protein, the MAPK. MAPKs represent a family of serine-threonine kinases with the potential to phosphorylate other cytoplasmic proteins and to translocate from the cytoplasm to the nucleus, where they can directly regulate the activity of transcription factors controlling gene expression.

MAPK signaling cascades are activated by several extracellular stresses such as ROS, UV, heat and oxidative stress in various cell types (Fig. 3)(Yoon et al., 2002). It is known that all these pathways contain some acceptable differences functions. ERK preferentially regulates cell growth and differentiation(Nishida and Gotoh, 1993; Marshall, 1995), and JNK and p38-MAPK function mainly in stress response(Derijard et al., 1994; Kyriakis et al., 1994; Raingeaud et al., 1995; Verheij et al., 1996). However,
Figure 3. The MAPK cascade in mammals. MAPK cascades consist of a module of three cytoplasmic kinases: a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK), an MAP kinase kinase (MAPKK), and the MAP kinase (MAPK) itself. Four major MAPKs, the ERK1/ERK2, JNK, ERK5 and p38-MAPK, are evolutionarily highly conserved enzymes connecting cell-surface receptors to critical regulatory target cascades within cells. (Yoon et al., 2002). MAPKs phosphorylate other cytoplasmic proteins and cause them translocate from the cytoplasm to the nucleus, where they can directly regulate the activity of transcription factors controlling gene expression. ERK preferentially regulates cell growth and differentiation(Nishida and Gotoh, 1993; Marshall, 1995), and JNK, p38-MAPK and ERK5 function mainly in stress response(Derijard et al., 1994; Kyriakis et al., 1994; Raingeaud et al., 1995; Verheij et al., 1996).
JNK and p38-MAPK can also be activated by mitogenic factors, such as epidermal growth factor and phorbol esters (Raingeaud et al., 1995). Thus JNK and p38-MAPK seem to act not only in apoptotic but also in mitogenic signaling. Recently, one study demonstrated that various sources of ROS increase cell proliferation, and JNK and p38-MAPK are rapidly and transiently activated by treatment with H$_2$O$_2$ (Kim et al., 2001). These observations suggest that MAPK signaling can be involved in the regulation of cell death and survival through phosphorylation cascade.
OBJECTIVE OF DISSERTATION

Oxidative stress is not only the major stress but also an inducer of many aging-related diseases. In order to recover from oxidative stress, organisms develop various rescue mechanisms to survive for millions of years. From worms to mammals, the PI3 kinase and MAPK signaling are two conserved evolutionary pathways involved in the oxidative stress response. Accumulating studies have demonstrated that phosphorylation cascades transducing cell death and survival signals to modulate cell fate. Like phosphorylation, O-GlcNAcylation is also able to modify small chemicals onto serine and threonine residues to modulate many nuclear and cytoplasmic protein functions. Therefore, it is easy to expect that O-GlcNAcylation has an inverse relationship with phosphorylation due to conformational inhibition (Zeidan and Hart). More interestingly, emerging research also point out that rapidly elevated total levels of O-GlcNAcylation is a critical survival signal for cells to resist various stresses. To date, there is some research to describing that O-GlcNAcylation modulates heat shock proteins to resist heat stress. However, the detailed relationship between oxidative stress and O-GlcNAcylation protection is relatively less understood. In the current dissertation, we use cell culture systems to determine the role of OGT on oxidative stress resistance. We also examine the effect of O-GlcNAcylation on the MAPK signaling and FOXO transcription factor which is negatively regulated by PI3 kinase signaling. Finally, we propose as our working model that OGT positively regulates FOXO4, a survival related protein, and negative modulates JNK1, a death related kinase, to resist oxidative stress.
O-GLCNAC TRANSFERASE MODULATES JNK1 TO RESIST ACUTE OXIDATIVE STRESS

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ABSTRACT

O-GlcNAcylation and phosphorylation are reciprocal in regulating cell function through attachment of O-GlcNAc or O-phosphate on the same or adjacent serine/threonine residues on proteins. Like phosphorylation, O-GlcNAcylation may participate in the cell survival process, by intensifying in response to a variety of stresses. However, the relationship between O-GlcNAcylation protection and oxidative stress is relatively unknown. In the current study, we show that stable O-GlcNAc transferase (OGT) cells are resistant to oxidative stress and have better recovery rate than stable nuclear cytoplasmic O-GlcNAcase and acetyltransferase (NCOAT) cells and control cells. Furthermore, OGT associates with the cell death-related kinase, c-Jun N-terminal kinase 1 (JNK1), under stress treatment. When cells are stressed with hydrogen peroxide, the O-GlcNAcylation of JNK1 obviates phosphorylation, thereby deactivating the death signal. The OGT-JNK1 pathway may provide a critical mechanism linking elevated O-GlcNAcylation levels with acute oxidative stress resistance.
INTRODUCTION

Oxidative stress is a major type of cellular stress that is defined as cellular and tissue damage resulting from the accumulation of free radicals under conditions where either the free radical formation is increased or the antioxidant defenses are inactivated (Halliwell, 1994; Betteridge, 2000). Basically, endogenous free radicals are generated as byproducts of common physiologic reactions and end products for specific immune defense purposes, and exogenous ones are generated from ozone, nitrogen dioxide, and electromagnetic radiation (Betteridge, 2000). Since superoxide dismutase was discovered in 1969, oxidative stress is a well-documented component of the aging process (Vincent et al., 2004) and several diseases such as diabetes mellitus (Mehta et al., 2006), cardiovascular disorders (Baker et al., 1998), neurodegenerative diseases (Lyras et al., 1997), autoimmune diseases (Mohan and Das, 1997), and muscular dystrophy (Ragusa et al., 1997).

It has been reported that evolutionally conserved mitogen-activated protein kinase (MAPK) is activated by oxidative stress and involved in the pathogenesis of many oxidative stress-mediated diseases. Four major MAPKs, the ERK1/ERK2, JNK, ERK5, and p38-MAPK, connect cell-surface receptors to critical regulatory targets cascades by phosphorylation within the cell. MAPK cascades consist of a module of 3 cytoplasmic
kinases: mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK) itself (Yoon et al., 2002). MAPKKK is a serine–threonine kinase that receives activating signals from a membrane-spanning receptor and then phosphorylates and activates its substrate, a MAPKK. This enzyme is a dual-specificity kinase with the potential to phosphorylate critical threonine and tyrosine residues in its substrate protein, MAPK. MAPKs represent a family of serine–threonine kinases with the potential to phosphorylate other cytoplasmic proteins and to translocate from the cytoplasm to the nucleus, where they can directly regulate the activity of transcription factors controlling gene expression. It is known that all these pathways contain some acceptable differences in function. ERK preferentially regulates cell growth and differentiation (Nishida and Gotoh, 1993; Marshall, 1995), and JNK and p38 MAPK function mainly in stress response (Derijard et al., 1994; Kyriakis et al., 1994; Raingeaud et al., 1995; Verheij et al., 1996). However, JNK and p38 MAPK can also be activated by mitogenic factors, such as epidermal growth factor and phorbol esters (Raingeaud et al., 1995). Thus, JNK and p38 seem to act not only in apoptotic but also in mitogenic signaling. Recently, one study demonstrated that various sources of ROS increase cell proliferation, and JNK and p38 MAPK are rapidly and transiently activated by treatment with H₂O₂ (Kim et al., 2001).
Oxidative stress-mediated diseases are also accompanied by elevated $O$-GlcNAcylation, which results in the modification of $O$-GlcNAc to serine and threonine residues on abundant cytoplasmic and nuclear proteins. As an important dynamic posttranslational modification, there is evidence showing that $O$-GlcNAcylation and $O$-phosphorylation reciprocally regulated many cellular functions by competitively modifying the same or adjacent sites and then altering the active state of phosphorylated proteins (Hart et al., 2007). The $O$-GlcNAc modification, discovered in 1984 (Torres and Hart, 1984), involved in the regulation multiple, diverse cell functions, including transcription, proteasomal degradation, proliferation, and apoptosis (Love and Hanover, 2005; Kudlow, 2006; Hart et al., 2007). Recently, Zachara et al., among others, further showed that $O$-GlcNAcylation may participate in the cell survival process, by intensifying in response to a variety of stresses (Zachara and Hart, 2004; Zachara et al., 2004; Champattanachai et al., 2006; Cieniewski-Bernard et al., 2006; Liu et al., 2006; Yang et al., 2006). However, the relationship between $O$-GlcNAcylation protection and oxidative stress is relatively unknown. Two enzymes, OGT and NCOAT, and substrate concentration UDP-GlcNAc resulting from glucose and glucosamine metabolism mainly regulates $O$-GlcNAcylation. Glucosamine, an $O$-GlcNAcylation activator, has been shown to provide benefits in several clinical disorders through the MAPK and other
pathways (Bissett, 2006). In addition, in animal models of alloxan (OGT inhibitor)- and streptozotocin (STZ; NCOAT inhibitor)-induce diabetes, MAPK activation is altered. Most importantly, the downstream effects of MAPK, c-Jun, c-Fos, and c-Myc have been shown to involve O-GlcNAcylation in many cell lines (Jackson and Tjian, 1988; Tai et al., 2004). Cheung et al also showed that OGT directly interacts with activated p38 through its C terminus and thus regulates O-GlcNAcylation of neurofilament H during glucose deprivation (Cheung and Hart, 2008). Since MAPK is involved in the pathogenesis of many oxidative stress-mediated diseases, it is very interesting and important to understand the role of O-GlcNAcylation in oxidative stress.

In the current work, we report that stable OGT-overexpressing cells are more resistant to hydrogen peroxide than control and stable NCOAT-overexpressing cells in a time- and dose-dependent manner. Moreover, hydrogen peroxide increases OGT-mediated JNK1 O-GlcNAcylation to prevent JNK1 phosphorylation. Altogether, our data provide a novel mechanism to link O-GlcNAcylation to oxidative stress resistance.
EXPERIMENTAL PROCEDURES

Plasmids—The plasmid p-EGFP-C1 (Clontech) was used to construct plasmids that express green fluorescence protein (GFP)-tagged fusion proteins. Rat OGT and mouse NCOAT were cloned to p-EGFP-C1 as described. Flag-tagged JNK1, Flag-tagged p38, and GFP-tagged ERK1 plasmid were generously supplied by Dr. Fang-Tsyrr Lin. 3xFlag–TEV–2xMyc tag was a kind gift from D. Crawford. All DNA was purified by cesium chloride gradient centrifugation followed by 1:1 phenol:chloroform extraction, ethanol precipitation, and resuspension in double distilled water.

Reagents and antibodies—Unless otherwise stated, all reagents were purchased from Sigma-Aldrich in USA. Protease inhibitor cocktail was purchased from Rouch Diagnostics (#11873580001). Lipofectamine 2000 reagent was purchased from Invitrogen in USA. G418 was purchased from Gibco/BRL. Anti-NCOAT (#345) and anti-OGT (AL28) antibody were kindly donated by Dr. Gerald Hart. Anti-ERK1 antibody was purchased from BD Transduction Lab (#610408). Anti-JNK2 (sc-7345), anti-p38 (sc-7149), and anti-GFP antibody (sc-8334) were purchased from Santa Cruz Biotechnology Inc. Anti-FLAG M2 antibody (F3165), Affinity Gel (A2220), and anti-OGT antibody (DM-17) were purchased from Sigma-Aldrich. Phospho-SAPK/JNK pathway sampler kit (#9912) and Phospho-MAPK Family antibody sampler kit (#9910)
were purchased from Cell Signaling.

**Cell Culture, transfection, and stable cell line establishment**—HEK 293 cells (ATCC) were maintained in DMEM media (Gibco) with 10% fetal bovine serum (Atlanta Biologicals) with 0.2 mg/mL penicillin and 0.4 mg/mL gentamicin according to standard procedures. HepG2 cells (ATCC) were grown under the same conditions with the addition of 1% non-essential amino acids (Gibco/BRL, Grand Island, NY). For the transient expression experiment, pEGFP-C1 empty, EGFP-tagged OGT, and EGFP-tagged NCOAT vectors were transfected into HEK 293 cells for 2 days by using Lipofectamine 2000 reagent. For stable cell line establishment, the transfected cells were further selected with 500 µg/mL G418 for 3 months and maintained in 200 µg/mL G418. The expression of EGFP, EGFP-OGT, and EGFP-NCOAT was examined and confirmed by Western blotting and fluorescence microscopy.

**Stress treatment, immunoprecipitation, and Western blotting**—Hydrogen peroxide was used as stress source. In general, cells were placed in a fresh medium 1 hour before the application of stress stimuli. Treatment conditions were dependent on the experiment needed. After stress treatment, the cells were washed with ice-cold PBS; harvested; and extracted with 1% NP-40 lysis buffer containing 50 mM Tris-HCl, 0.5 M NaCl, 20% glycerol (v/w), 5 mM MgCl₂, 0.2 mM EDTA (pH 8.0) plus proteases inhibitor cocktail
and 0.5 mM orthovanadate. Co-immunoprecipitations were performed as previously described (Yang et al., 2001). Precipitation was carried out using ANTI-FLAG M2 affinity gel or antibody followed by treatment with 1:1 protein A:protein G beads (Amersham). Extracts were separated by 10% noncontinuous reducing SDS-PAGE on Tris-glycine gels. Proteins transferred to nitrocellulose and blocked with 5% (w/v) non-fat milk and detected with specific antibodies. Densitometry was performed using nonsaturated chemiluminescent exposed films and quantitated using ImageJ free software analyzer (NIH, http://rsb.info.nih.gov/ij/).

Cell survival—Media were changed 1 h before the initiation of oxidative stress-survival experiments. Cells were treated with hydrogen peroxide at different range of concentrations for 1 hour or with 0.3 mM for the indicated time and then recovered for 16 or 24 hours. The cell number and survival rate were examined using a Coulter counter as previously described. Cell viability was determined using trypan blue and presented as a percentage. To allow for differences in growth rates derived from OGT and NCOAT levels, cell viability was expressed as a percentage of unstressed cells. All experiments were performed a minimum of 3 times, and the numbers are derived from at least 2 replicates. Error bars represent 1 SD; \( p \) values are the result of a paired Student \( t \) test (two-tailed).
RESULTS

Establishment of stable EGFP-OGT- and EGFP-NCOAT-overexpressing HEK 293 cell lines—In order to directly investigate the roles of OGT and NCOAT under oxidative stress, we established stable EGFP-tagged OGT and EGFP-tagged NCOAT HEK 293 cells (Fig. 1). The stable cell lines were prepared as described in “Experimental Procedures”, and checked using gfp-fluorescence microscopy (Fig. 1A), and confirmed using Western blotting (Fig. 1B). Compared with HEK 293 cells, stable EGFP, EGFP-OGT, and EGFP-NCOAT cell lines appeared green under fluorescence microscopy. Consistent with previous studies, EGFP was expressed throughout HEK 293 cells, and EGFP-OGT and EGFP-NCOAT were predominantly localized in the cytoplasm (Kreppel et al., 1997; Lubas et al., 1997; Gao et al., 2001) (Fig. 1A).

EGFP-OGT and EGFP-NCOAT expression were further confirmed using Western blotting with anti-EGFP, anti-OGT, and anti-NCOAT antibodies. Three bands, corresponding to the molecular masses of EGFP, EGFP-OGT, and EGFP-NCOAT (approximately 30 kD, 130 kD, and 150 kD, respectively), were detected (Fig. 1B, panel 1). Anti-OGT and anti-NCOAT antibodies indicated that the 130-kD and 150-kD bands were EGFP-OGT and EGFP-NCOAT respectively (Fig. 1B, panels 2 and 3). Compared with HEK 293 and other stable cell lines, the stable EGFP-OGT cell line showed an
approximately 1.5-fold increase in the amount of total OGT protein; the stable
EGFP-NCOAT cell line showed an approximately 2.2-fold increase in the total NCOAT
protein amount (Fig. 1C). Notably, EGFP-NCOAT overexpression resulted in an about
0.2-fold reduction in endogenous OGT expression in the stable NCOAT cell line.
However, EGFP-OGT overexpression did not affect endogenous NCOAT expression.
EGFP overexpression did not change the expression level of endogenous OGT and
NCOAT (Fig. 1C).
Studies have shown that transient overexpression of OGT and NCOAT in mammalian
cells dramatically increases and decreases O-GlcNAcylation levels respectively (Kreppel
et al., 1997; Lubas et al., 1997; Gao et al., 2001). Therefore, we also evaluated the effect
of overexpression of EGFP-OGT and EGFP-NCOAT on total O-GlcNAcylated proteins
in stable cell lines. The total extracted proteins of the 4 cell lines were examined using a
specific anti-O-GlcNAc monoclonal antibody (Fig. 1B, panel 4). Compared with HEK
293 and stable EGFP cell lines, the stable EGFP-NCOAT cell line showed an about
0.5-fold reduction on total O-GlcNAcylated protein levels. However, the stable
EGFP-OGT cell line did not show any change in the level of total O-GlcNAcylated
proteins (Fig. 1C). Taken together, these data indicate successful overexpression of
EGFP-tagged OGT and NCOAT in HEK 293 cells.
Figure 1

(A) EGFP, EGFP-OGT, EGFP-NCOAT

(B) WB:

- EGFP-NCOAT/ 150 kD
- EGFP-OGT/ 130 kD
- EGFP/ 30 kD
- EGFP-OGT/ 130 kD
- OGT/ 110 kD
- EGFP-NCOAT/ 150 kD
- NCOAT/ 135 kD
- O-GlcNAcylated proteins
- ACTIN

(C)

- OGT Level
- NCOAT Level
- O-GlcNAcylated Protein Level

Relative amount of total OGT (Fold)

Relative amount of total NCOAT (Fold)

Relative amount of total O-GlcNAcylated proteins (Fold)

*P<0.05
FIGURE 1. Establishment of EGFP-tagged OGT and NCOAT stable HEK 293 cell lines. EGFP empty, EGFP-tagged OGT, and NCOAT vectors were transfected into HEK 293 cells and selected using G418 for 3 months. A, Stable EGFP empty vector (left column), EGFP-tagged OGT vector (middle column), EGFP-tagged NCOAT vector (right column) cell lines were examined by fluorescence microscopy in 63× amplified fields. The middle panel was stained with the anti-\(O\)-GlcNAc antibody RL2. B, Extracts (50 µg detergent) of every stable cell line were blotted with anti-OGT, anti-EGFP, anti-NCOAT, anti-\(O\)-GlcNAc, and anti-actin antibodies by using western blotting. C, Quantification of total OGT (left panel), NCOAT (middle panel), and total \(O\)-GlcNAcylated proteins (right panel) in Figure 1B by using the ImageJ software tool. Relative amounts of total OGT, NCOAT, and \(O\)-GlcNAcylated proteins, and all protein levels are compared with control HEK 293 cells and expressed as the fold concentrations in control cells.
**Effect of hydrogen peroxide treatment on stable EGFP-OGT and EGFP-NCOAT cell lines**—To examine the effect of oxidative stress on stable EGFP-OGT and EGFP-NCOAT cell lines, the cells were treated with hydrogen peroxide in a time- and dose-dependent manner. The cell recovery rate was determined by trypan blue staining (Fig. 2A&B). When cells were challenged with different concentrations of hydrogen peroxide, the stable EGFP-OGT cell line showed a higher survival rate than that of HEK 293 cells and control EGFP cell lines in a time- and dose-dependent manner. In contrast, the stable EGFP-NCOAT cell line showed a significant reduction in survival rate when compared to the control cell lines. The higher survival rate of stable EGFP-OGT cells was confirmed by caspase 3-mediated PARP-1 cleavage (Fig. 2C). Compared with the control and EGFP-NCOAT cells, EGFP-OGT cells showed a dramatic reduction in the amount of PARP-1 cleavage products (Fig. 2C). Further, we also checked the effect of oxidative stress on total O-GlcNAcylated protein levels in EGFP, EGFP-OGT, and EGFP-NCOAT cells. The stable cell lines were treated with 0.5 mM hydrogen peroxide for 24 hours, and total O-GlcNAcylated proteins were analyzed by western blotting. All cell lines, except EGFP-NCOAT, seem to show an elevation in the total O-GlcNAcylated protein levels (data not shown). These data suggest that OGT overexpression can prevent oxidative stress-mediated cell death in HEK 293 cells. Therefore, it is very important to
Figure 2

(A) Cell viability (%) vs. \( \text{H}_2\text{O}_2 \) concentration (mM)

- **EGFP**
- **EGFP-OGT**
- **EGFP-NCOAT**
- **293**

1 hour
Dose course

- #p<0.05
- *p<0.005

(B) Cell viability (%) vs. time (hours)

- **H2O2 (300 µM)**

Time course

- #p<0.05
- *p<0.005

(C) Western Blot Analysis

<table>
<thead>
<tr>
<th>H2O2, 100 µM 24hr</th>
<th>EGFP</th>
<th>EGFP-NCOAT</th>
<th>EGFP-OGT</th>
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- **αPARP-1**
- **αACTIN**

- PARP-1/116 kD
- Cleaved PARP-1/89 kD
- ACTIN
FIGURE 2. Effect of hydrogen peroxide treatment on EGFP-OGT and EGFP-NCOAT stable cell lines. A&B, The stable cell lines were treated with 0.3 mM hydrogen peroxide for different times and with different doses of hydrogen peroxide for 1 hour. The cell recovery rate was determined by trypan blue staining. C, The stable cell lines were treated with 0.1 mM hydrogen peroxide for 24 hours, and PARP-1 cleavage (top panel) and actin (bottom panel) were analyzed using western blotting.
examine the mechanisms linking O-GlcNAcylation with oxidative stress-dependent activation of death-related pathways.

*Oxidative stress fails to induce JNK1 phosphorylation in stable EGFP-OGT cells*—To determine whether oxidative stress-mediated MAPK signaling affects stable cell lines, cells were treated with hydrogen peroxide. Phosphorylation of ERK1/2, p38, and JNK1 was analyzed using Western blotting (Fig. 3). Hydrogen peroxide induced phosphorylation and activation of ERK1/2, p38, and JNK1 in HEK 293 cells (Fig. 3, left column). It also resulted in the phosphorylation of ERK and p38 in the stable EGFP-OGT cell line. However, hydrogen peroxide failed to induce phosphorylation of JNK1 at 1 hour incubation (Fig. 3, middle panel) in the stable EGFP-OGT cell line. A similar result was also obtained upon oxidative stress treatment of cells showing transient OGT overexpression (Fig. 4A, panel 3). In contrast to stable EGFP-OGT cells, EGFP-NCOAT cells showed phosphorylation of ERK, p38, and JNK1 in response to hydrogen peroxide (Fig. 3, right panel). Surprisingly, oxidative stress did not induce greater levels of JNK1 phosphorylation level in the stable EGFP-NCOAT cell line than in HEK 293 cells. Zachara et al showed that upregulation of both OGT activity and NCOAT activity occurs upon heat stress stimulation; however, they stated that OGT activity is a major rescuer of
Figure 3

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<th>Western blot</th>
<th>293 cells</th>
<th>OGT stable</th>
<th>NCOAT stable</th>
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<tr>
<td>H₂O₂ 0.5 mM, 1hr</td>
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<td>αEGFP</td>
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<td>αp-JNK1</td>
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**FIGURE 3. Effect of hydrogen peroxide treatment on phosphorylation of MAPKs in stable EGFP-OGT and NCOAT cell lines.** HEK 293 cells (left column), and stable EGFP-OGT (middle column) and EGFP-NCOAT (right column) 293 cells were treated with or without 0.5 mM hydrogen peroxide for 1 hour. After treatment, the 50 µg detergent extracts of every cell line were blotted with anti-EGFP, anti-OGT, anti-actin, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-JNK1, and anti-JNK1 antibodies. All experiments were performed at least 3 times.
the increase in the amount of total O-GlcNAcylated protein (Zachara et al., 2004). We also found that OGT overexpression affected hydrogen peroxide-induced JNK1 nucleus translocation in HEK 293 cells (data not shown). Our data suggest that OGT is specifically involved in the inhibition of oxidative stress-mediated phosphorylation of JNK1.

*OGT associates with JNK1 upon hydrogen peroxide treatment in vivo*—OGT can directly and indirectly associate with and interrupt many proteins through its N-terminal TPR domain (Kreppel and Hart, 1999; Lubas and Hanover, 2000; Beck et al., 2002; Yang et al., 2002; Iyer et al., 2003; Iyer and Hart, 2003a, b; Jinek et al., 2004). To investigate how OGT affects hydrogen peroxide-mediated JNK1 phosphorylation and nucleus translocation, we tested the possibility of interaction between OGT and JNK1 in vivo and in vitro (Fig. 4). HEK 293 cells were transfected with Flag-tagged JNK1, p38, or OGT expression vector in the presence or absence of EGFP-tagged OGT or ERK1 expression vector. The transfected cells were treated with 0.5 mM hydrogen peroxide for 1 hour and washed twice with iced PBS. Co-immunoprecipitation was performed using anti-Flag M2 beads and anti-EGFP antibody, and the cells were then immunoblotted with anti-EGFP or anti-Flag antibodies. As seen in Fig. 4, EGFP-OGT proteins were only detected in cells
Figure 4  (A)

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<tr>
<td>Flag-OGT</td>
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(B)

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αOGT  
αJNK1  
OCT/ 110 kD  
JNK1/ 54 kD  
JNK1/ 46 kD  
IgG heavy chain
FIGURE 4. OGT associates with JNK1 upon hydrogen peroxide treatment. A, HEK 293 cells were co-transfected with the indicated expression vectors for 2 days. The transected cells were treated with 0.5 mM hydrogen peroxide for 1 hour, and the detergent extracts were immunoprecipitated with anti-Flag M2 beads and then analyzed using western blotting with anti-EGFP, anti-Flag, anti-p-JNK1, and anti-p-p38 antibodies. Ten percent of inputs were immunoblotted with anti-EGFP and anti-Flag antibodies for control of protein expression (panels 5 and 6). The star indicates a non-specific band. B, In HEK 293 cells, endogenous OGT and JNK1 were directly interacted by using anti-JNK1 antibody for immunoprecipitation and analyzed using western blotting with anti-OGT and anti-JNK1 antibodies. The detergent extracts were immunoprecipitated with normal mouse IgG as immunoprecipitation controls (right). The 50 µg detergent extracts were blotted with anti-JNK1 and anti-OGT antibodies as protein-expression controls (right column).
immunoprecipitated with Flag-JNK1 after hydrogen peroxide treatment (Fig. 4A, top panel). We did not detect an increase in the association between OGT and p38 or ERK1 under these conditions. Consistent with the results depicted in Fig. 3, OGT prevented hydrogen peroxide-mediated phosphorylation of JNK1 but not of p38 (Fig. 4A, panels 3 and 4). Ten percent of the input is shown in the bottom panel of Fig. 4A. These data and those in Fig. 3 suggest that OGT is specifically involved in the regulation of JNK1 rather than other MAPKs activated by oxidative stress.

To further confirm the interaction between endogenous OGT and JNK1, HEK 293 cells were directly immunoprecipitated with anti-JNK1 antibody (Fig. 4B). Consistent with the results of co-immunoprecipitation (Fig. 4A), hydrogen peroxide increased the amount of OGT in anti-JNK1 antibody-precipitated cells. The IgG-immunoprecipitated control cells and 50 µg whole cell lysates are shown in the bottom panel of Fig. 4. Taken together, OGT associates with JNK1 after oxidative stress stimulation.

O-GlcNAcylation and phosphorylated JNK1 have an inverse relationship in hydrogen peroxide-treated HEK 293 cells—O-GlcNAcylation and phosphorylation can potentially regulate cell function through the reciprocally attachment of O-GlcNAc or O-phosphate to the same or adjacent serine and threonine residues on proteins(Hart et al.,
Oxidative stress can induce JNK1 phosphorylation at Thr183 and Tyr185, which are 2 important residues for the function of upstream kinases such as MKK4/7 (Lawler et al., 1998; Lisnock et al., 2000). We used the “Ying-Yang” program (http://www.cbs.dtu.dk/services/YinOYang/) to predict the $O$-GlcNAcylation sites of JNK1 and found that Thr183 and Thr188 are likely to be $O$-GlcNAcylated. Here, we further examined the $O$-GlcNAcylation of JNK1 in HEK 293 cells (Fig. 5). EGFP-tagged OGT and Flag-tagged JNK1 vectors were co-transfected into HEK 293 cells for 48 hours. The transfected cells were treated with 1 mM hydrogen peroxide for 1 hour. The cell extracts were immunoprecipitated with anti-Flag antibody and analyzed using western blotting with anti-JNK1, anti-phospho-JNK1, and anti-$O$-GlcNAc antibodies. Consistent with the results shown in figure 4, oxidative stress increased the EGFP-OGT protein level in Flag-tagged JNK1 precipitates (Fig. 5, panel 1). Overexpression of Flag-tagged JNK1 seems to cause its phosphorylation even in the presence of overexpression of EGFP-tagged OGT. However, oxidative stress caused de-phosphorylation of JNK1 in the presence of overexpression of EGFP-tagged OGT (Fig. 5, panel 3). Surprisingly, oxidative stress resulted in JNK1 $O$-GlcNAcylation, but not in the normal condition, as detected using anti-$O$-GlcNAc antibody detection (Fig. 5, panel 2). The amount of phosphorylated JNK1 and $O$-GlcNAcylated JNK1 after hydrogen peroxide treatment
FIGURE 5. $O$-GlcNAcylated and phosphorylated JNK1 have an inverse relationship in hydrogen peroxide-treated HEK 293 cells. EGFP-tagged OGT and Flag-tagged JNK1 vectors were co-transfected into HEK 293 cells for 48 hours. The transfected cells were treated with 1 mM hydrogen peroxide for 1 hour. The cell extracts were immunoprecipitated with anti-Flag antibody and analyzed using western blotting with anti-EGFP (panel 1), anti-$O$-GlcNAc (panel 2), anti-p-JNK1 (panel 3), and anti-Flag (panel 4) antibodies. Ten percent of the input was immunoblotted with anti-EGFP and anti-Flag antibodies as protein-expression controls (panels 5 and 6).
were inversely related. The 10% input is shown in the bottom panel of Fig. 5. These results show that oxidative stress promotes OGT-JNK1 association and that JNK1 O-GlcNAcylation may interrupt JNK1 phosphorylation.

\textit{O-GlcNAcylation prevents JNK1 phosphorylation upon hydrogen peroxide treatment in HepG2 cells}—In order to show the dynamic nature of the O-GlcNAc modification of JNK1 upon oxidative stress treatment, HEK 293 cells were treated with 1 mM hydrogen peroxide for varying times (Fig. 6). Because specific anti-O-GlcNAcylated antibodies for JNK1 and OGT were not available, the cell lysates were immunoprecipitated with a specific anti-O-GlcNAc antibody and then immunoblotted with anti-JNK1 and anti-OGT antibodies to identify target O-GlcNAcylated proteins (Fig. 6, panels 1 and 2). OGT is a well-known positive control for O-GlcNAcylated proteins (Kreppel \textit{et al.}, 1997). As seen in Fig. 6, only 1 clear OGT band was detected in the anti-O-GlcNAc immunoprecipitated cells at all times compared to 2 OGT bands in the whole cell lysates (Fig. 6, panels 3 and 4). Oxidative stress did not affect the O-GlcNAcylation level of OGT. Surprisingly, O-GlcNAcylated JNK1 appeared at 30 min and peaked at 60 min then diminished with increasing H$_2$O$_2$ incubation (Fig. 6, panel 1). Identical results were also obtained in HepG2 cells (data not shown). On the basis of the
FIGURE 6. Hydrogen peroxide induces dynamic O-GlcNAcylation of JNK1 in HEK 293 cells. HEK 293 cells were treated with hydrogen peroxide at the indicated times, then immunoprecipitated with anti-O-GlcNAc antibody and blotted with anti-JNK1 (panel 1) and anti-OGT (panel 2) antibodies. The 50 µg detergent extracts were blotted with anti-JNK1 (panel 3), anti-OGT (panel 4), and anti-actin antibodies. One hour after the treatment, the detergent extracts were immunoprecipitated with normal mouse IgG as immunoprecipitation controls.
above evidence (Figs. 2, 3, 4, and 5), OGT may dynamically interrupt JNK1 phosphorylation to resist oxidative stress.
DISCUSSION

Accumulating evidence points to the essential roles of O-GlcNAc modification in regulating diverse functions of nuclear and cytoplasmic proteins (Love and Hanover, 2005; Kudlow, 2006; Hart et al., 2007). Our study provides the first evidence that links this modification and MAPK-signaling events to resistance to oxidative stress and shows two important posttranslational modifications, O-GlcNAcylation and O-phosphorylation, of JNK1 as a critical factor for cell fate after this stress treatment. JNK1 activation plays a central role in many cellular processes and diseases, so the interaction between OGT and JNK1 opens up a new regulators mechanism for the pathogenesis of many chronic diseases.

Increase in O-GlcNAc modification seems to play two controversial functions in cells. On the one hand, the increasing level of O-GlcNAc modification has been reported in some glucose metabolism-related chronic diseases (Dias and Hart, 2007; Hart et al., 2007; Zhang et al., 2007) as well as in the aging of tissues (Fulop et al., 2008). On the other hand, Zachara et al, among others, have shown that increasing the level of O-GlcNAc modification provides a protective mechanism against various stresses (Zachara et al., 2004; Jones et al., 2008) and may involve heat shock proteins (Guinez et al., 2004). In the current study, we further show that OGT directly associates
with JNK1 to prevent the latter’s activation and then resists oxidative stress. Because oxidative stress occurs throughout the cells at all times, we hypothesize that the increase in O-GlcNAc modification in aging tissues might be a byproduct of this or other stresses. Activation followed by termination of signal transduction is essential for all signaling pathways. O-GlcNAcylation is mainly regulated by two enzymes, OGT/NCOAT, and by the concentration of UDP-GlcNAc, which is formed during glucose and glucosamine metabolism. OGT activity can be regulated by tyrosine phosphorylation, another posttranslational modification participating in the cellular response to stressful stimuli (Song et al., 2008). NCOAT, however, has been reported to be involved in two posttranslational modifications as well as cleavage by caspase 3, but the mechanism regulating its activity is unknown (Gao et al., 2001; Wells et al., 2002; Lazarus et al., 2006; Butkinaree et al., 2008). In figure 1 B, C, we show that both OGT and NCOAT protein levels undergo a 1.5- and 2.2-fold increase compared to control and stable EGFP-empty cells. Also, NCOAT overexpression seems to result in downregulation of OGT expression in the long term. Stable OGT, but not NCOAT, cell line shows a compensation feedback downregulation of O-GlcNAcylation level to the basal level as in the control cells and under the normal condition (Fig. 1C). However, stable OGT cells still have the highest survival rate upon stress treatment (Fig. 2). These data indicate that
OGT and NCOAT activity are tightly regulated by an unknown mechanism and play a key role in stress response. The increasing O-GlcNAc modification in aging-related diseases might be lost upon NCOAT activity feedback regulation after oxidative stress stimulation.

NCOAT has been reported to be show a strong genetic association with diabetes in Pima Indians and Mexican Americans (Farook et al., 2002; Lehman et al., 2005) as well as in late-onset Alzheimer’s disease (Bertram et al., 2000). Some alternative splicing variants of NCOAT have also been cloned from brain tumors (Comtesse et al., 2001) and spontaneous diabetes GK mice (Whisenhunt et al., 2006). Our laboratory has successfully used a inducible transgenic mouse system to overexpress dominant negative activity of NCOAT and demonstrate the symptoms of aging-related diseases in many tissues (Bowe et al., 2006; Wang et al., 2009). Taken together, O-GlcNAcylation levels seems to be precisely and tightly maintained in a basal range, which is coupled with the nutrition level and blood glucose concentration under normal physiology.

O-GlcNAcylation level is dynamically regulated in response to various acute stress stimuli through OGT and NCOAT activity and UDP-GlcNAc concentration. Here, we show that stable OGT stable cells are resistant to oxidative stress and have better recovery rate than stable NCOAT cells and control cells. Furthermore, oxidative stress
increases OGT association with JNK1. When 293 cells and HepG2 cells are stressed with hydrogen peroxide, the O-GlcNAcylation of JNK1 obviates phosphorylation thereby deactivating the death signal. Our studies show that the OGT-JNK1 pathway may provide a critical mechanism linking elevated O-GlcNAcylation levels with acute oxidative stress resistance.
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O-GLCNACYLATION ENHANCES FOXO4 TRANSCRIPTIONAL REGULATION IN RESPONSE TO STRESS

by

SHIUH-RONG HO, KAI WANG, THOMAS R. WHISENHUNT, PING HUANG, XIAOLIN ZHU, JEFFREY E. KUDLOW, AND ANDREW J. PATERSON


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ABSTRACT

The FOXO4 transcription factor plays an important role in cell survival in response to oxidative stress. The regulation of FOXO4 is orchestrated by post-translational modifications including phosphorylation, acetylation, and ubiquitination. Here, we demonstrate that O-GlcNAcylation also contributes to the FOXO4-dependent oxidative stress response. We show that hydrogen peroxide treatment of HEK293 cells increase FOXO4 association with OGT, the enzyme that adds O-GlcNAc to proteins, causing FOXO4 O-GlcNAcylation and enhanced transcriptional activity under acute oxidative stress. O-GlcNAcylation is known to be protective of cells under stress conditions, including oxidative stress. Our data provide a mechanism of FOXO4 anti-oxidative protection through O-GlcNAcylation.
1. INTRODUCTION

FOXO4, together with FOXO1, FOXO3 and FOXO6, belongs to a subset of the Forkhead family of transcription factors [1]. Like other Forkhead box O (FOXO) transcription factors, FOXO4 is able to modulate the expression of genes involved in oxidative stress dependent apoptosis, cell cycle arrest, DNA damage repair and other cellular functions [2,3]. Essers et al showed FOXO4 proteins reduce oxidative stress by directly increasing mRNA and protein levels of manganese superoxide dismutase (MnSOD) and catalase [4]. Furthermore, FOXO4 can up-regulate the cyclin-dependent kinase inhibitor gene, p27\textsuperscript{Kip1}, causing cell cycle arrest, DNA repair, and is thus tumor suppressive [5]. When ineffective DNA repair or excessive damage ensues, cell death is triggered. Thus, FOXO4 may function as a switch between cell death and survival under oxidative stress stimulation.

The FOXO4 transcription factor is regulated in cellular shuttling and DNA binding of target genes by multiple mechanisms. Normally, AKT negatively regulates all FOXO protein activity by direct phosphorylation, causing their nuclear exclusion [6]. However, oxidative stress causes nuclear localization of FOXO4 through Mdm2-dependent mono-ubiquitination [7] and resultant transcriptional activation increase through JNK-mediated phosphorylation at residues 471 and 451 [4]. Furthermore, the longevity protein, SIRT1, deacetylates FOXO proteins and modulates their transcriptional activity in response to stress stimuli [8,9]. Housley and others also found that FOXO1 is O-GlcNAcylated in response to glucose [10,11]. However, O-GlcNAcylaion of FOXO4 under oxidative stress has not been studied.
O-GlcNAcylation is an abundant and dynamic post-translational modification (PTM) on serine and threonine residues of nuclear and cytoplasmic proteins. O-GlcNAc Transferase (OGT) and Nuclear Cytoplasmic O-GlcNAcase and Acetyltransferase (NCOAT) catalyze this process of adding and removing the O-GlcNAc groups respectively. O-GlcNAcylation plays a critical role in protein turnover, cell cycle progression, transcription, stress response and other cellular functions [12-14]. Zachara and others showed that a global increase in protein O-GlcNAc levels is critical for cell survival in response to oxidative stress and other stimuli [15,16]. However, a mechanism explaining how O-GlcNAcylation contributes to the oxidative stress response remains complicated.

The regulation of FOXO4 in response to oxidative stress involves many PTMs that include, phosphorylation, acetylation and ubiquitination. Here, we show that FOXO4 is also O-GlcNAcylated and that this modification is increased upon acute oxidative stress treatment. Furthermore, O-GlcNAc modification causes increased transcriptional activity of FOXO4 in stress-related genes. Our work provides another mechanism whereby FOXO4 contributes to the anti-oxidative stress process, through the modulation of O-GlcNAcylation.
2. MATERIALS and METHODS

2.1. Cell culture, plasmids and transfection

HEK293 and HepG2 cells were maintained in Dulbecco's Modified Eagle medium (1g/L glucose) (Cellgro), supplemented with 10% fetal bovine serum and penicillin/streptomycin. Rat OGT and mouse NCOAT were inserted into pEGFP-C1 (Clontech) as previous described [17]. Mouse FLAG-FOXO4 and myc-FOXO4 plasmids were gifts from Dr. Zhi-Ping Liu [18]. 6X daf-16 protein binding element (DBE)-Luc [19] was a gift from Dr. Boudewijn Burgering. The p27Kip1-DBE-Luc was a gift from Dr. Simon Lees [20]. Transient transfections were performed with Lipofectamine 2000 according to the manufacturer (Invitrogen).

2.2. Transcription activation assay

HEK293 cells were transfected with the indicated constructs plus 10 ng TK-Renilla (Promega) used as a transfection efficiency control. Cells were treated with 200 µM H₂O₂ for an hour and lysates were measured by the Promega Dual Luciferase reporter assay.

2.3. Protein interaction analysis

Co-immunoprecipitation (co-IP) or IP assays were performed on lysates from HEK293 cells transfected with EGFP-OGT and FLAG-FOXO4 or HepG2 cells. Anti-FLAG M2 Affinity Gel (Sigma) and anti-OGT antibody were used in this Co-IP and IP assay. The immunoprecipitates were washed five times and separated by 10% SDS-PAGE gel electrophoresis and blotted using anti-EGFP (Santa Cruz), anti-FLAG-HRP, anti-O-GlcNAc (RL2, or CTD110.6, Covance), anti-p(Ser193)-FOXO4 (Cell Signaling), anti-OGT (DM-17, Sigma), anti-GAL4 (sc-577), anti-FOXO4 and anti-Nucleolin (Santa
2.4. Western blot analysis

\(O\)-GlcNAcylated proteins were immunoprecipitated using anti-\(O\)-GlcNAc antibody and then immunoblotted with anti-FLAG (Sigma), anti-\(\alpha\)-TUBULIN (Sigma), anti-FOXO4 (Santa Cruz), anti-FOXO1 (Santa Cruz and Abcam), anti-FOXO3 (Santa Cruz and Abcam) and anti-SIRT1 (Santa Cruz).

2.5. Immunofluorescence

Mouse FLAG-FOXO4 was transfected into HEK293 cells and maintained at normal growth medium for 24 hours. Media was refreshed an hour before the administration of glucosamine, TT06 and LY294002 (OGT and AKT inhibitors). Cells were treated with 500 \(\mu\)M \(H_2O_2\) for another hour then fixed with 4\% paraformaldehyde and stained with anti-FLAG-cy3 antibody and DAPI.

2.6. Statistical analysis

All experiments were performed at least three times. Data are presented as mean ± S.E. and represent three independent experiments. Student’s \(t\)-test was using for comparing group means, and P values \(\leq 0.05\) were accepted as significant.
3. RESULTS

3.1 Oxidative stress modulates FOXO4 protein and O-GlcNAc levels

We first needed to determine if stress stimuli effected O-GlcNAc modification on FOXO proteins. The treatment of transiently transfected Flag-tagged FOXO4 HEK293 cells with hydrogen peroxide resulted in a dramatic increase in O-GlcNAc-modified FOXO4 levels (Fig. 1A). Interestingly, H₂O₂ treatment of the HEK293 cells reduced total FOXO4 protein levels in the 10% extract. Similarly, heat shock stress also produced a progressive increase in O-GlcNAcylated endogenous FOXO4 protein in HepG2 cells (Fig. 1B and C). Specificity was confirmed by free GlcNAc competition. Although FOXO1 has been reported to be O-GlcNAcylated in response to glucose treatment [10,11], we did not detect O-GlcNAc-FOXO1 in heat-stressed cells. The longevity protein, SIRT1, also remained unchanged in O-GlcNAc and total protein levels (Fig. 1B). Not only is FOXO4 O-GlcNAc-modified in HepG2 cells, but OGT is physically associated with FOXO4 under heat stress (Fig. 1D).

3.2 OGT interacts and O-GlcNAcylates FOXO4 under oxidative stress

The acute oxidative stress-mediated O-GlcNAcylation of FOXO4 was accentuated with the association between FOXO4 and OGT, demonstrated here in HEK 293 cells in a co-IP assay (Fig. 2). Hydrogen peroxide induced significant binding of transfected EGFP-tagged OGT protein with immunoprecipitated FLAG-tagged FOXO4 (Fig. 2A, panel 1). The O-GlcNAcylation of FOXO4 proteins was confirmed with O-GlcNAc antibody, using 1 M GlcNAc as an O-GlcNAc specific competitor control (Fig 2A, panels 4 and 5).
Fig. 1. **O-GlcNAcylation of FOXO4 protein with H₂O₂.** (A) Western blot of HEK293 cells transfected with FLAG-tagged FOXO4, 24 hours later treated with 500 µM H₂O₂ for 60 minutes. Cell lysates were immunoprecipitated with antibody (RL2), separated on SDS-PAGE, and immunoblotted with anti-FLAG or anti-FOXO4 antibodies. Total protein levels of FOXO4 and tubulin control are shown with 10% input. (B) HepG2 cells stressed at 42°C for indicated times and lysates IP’d with anti-O-GlcNAc, and immunoblotted with FOXO1a, FOXO4, and SIRT1 antibodies. (C-D) HepG2 cells stressed at 42°C for 2 hours, lysates IP’d with anti-FOXO4 or anti-OGT, and immunoblotted with O-GlcNAc or FOXO4 antibodies.
Immunoprecipitation blots with OGT deletion constructs indicated that FOXO4 protein principally binds to the C-terminus of OGT (OGT a.a. 486-944) (Fig. 2C).

It has been reported that O-GlcNAcylation may have a reciprocal role with phosphorylation [12]. AKT-mediated phosphorylation of FOXO4 at Ser193 regulates its nuclear exclusion, thus negatively regulating transcription of its target genes. Treatment with H_2O_2 resulted in an 80% elevation of O-GlcNAcylated FOXO4 (Fig. 2B), with a corresponding 20% reduction in Ser193-specific phosphorylation. However, since the number of potential O-GlcNAc sites on FOXO4 is unknown, reciprocity between phosphorylation and O-GlcNAcylation cannot be directly compared. Our data have shown that acute H_2O_2 induces OGT-FOXO4 association with O-GlcNAc modification of FOXO4, and oxidative stress also causes a reduction of AKT-dependent phosphorylation of FOXO4 with increased nuclear FOXO4 protein [6]. We now consider if FOXO4 cellular localization is O-GlcNAc-dependent.

3.3. Modulation of O-GlcNAcylation does not affect redistribution of FOXO4 proteins

To investigate the effect of modulation of O-GlcNAcylation on FOXO4 cellular localization, HEK293 cells transfected with FLAG-tagged FOXO4 plasmid were treated with glucosamine and the AKT inhibitor, LY294002, before H_2O_2 treatment. The increase in total cellular protein-O-GlcNAc was confirmed by Western blot (data not shown). FOXO4, normally distributed throughout the cytoplasm, was largely restricted to the nucleus upon H_2O_2 treatment (Fig. 3, panel 1). Control cells treated with the AKT inhibitor, LY294002, also showed FOXO4 confined to the nucleus, inferring that AKT activity is required to keep FOXO4 in the cytoplasm (Fig. 3, panel 3). However, increased O-GlcNAcylation by glucosamine treatment did not promote nuclear localization.
**Fig. 2. OGT interacts and O-GlcNAcylates FOXO4.** (A) Western blot of HEK293 cells that were co-transfected with FLAG-FOXO4 and EGFP-OGT for 24 hours and treated with 500 µM H₂O₂ for 60 min. Cell lysates were IP’d with anti-FLAG, separated on SDS-PAGE, and immunoblotted with GFP, FLAG, and p(Ser193) FOXO4 antibodies or anti-O-GlcNAc ± 1 M GlcNAc. (B) Relative Ser193 phosphorylation and O-GlcNAcylation of FLAG-tagged FOXO4 of Figure 2A analyzed with “ImageJ”. All values expressed as means ± S.E. for three independent experiments. *P≤0.05 vs. control. (C) Western blot of HEK293 cells that were co-transfected with FLAG-FOXO4 and indicated GAL4-tagged OGT deletion constructs for 24 hours and treated with 500 µM H₂O₂ for 60 min. Cell lysates were IP’d with anti-FLAG, and immunoblotted with FLAG and GAL4 antibodies.
Fig. 3. FOXO4 redistribution, unaffected by O-GlcNAcylation. Immunofluorescence of HEK293 cells transfected with FLAG-FOXO4 and treated with 20 mM glucosamine or 20 µM LY294002 for 60 minutes then stimulated with 500 µM H₂O₂ for 60 minutes. Cells were fixed and stained with anti-FLAG-cy3 targeting FOXO4 (red), and DAPI for nucleus (blue).
localization of FOXO4 (Fig. 3, panel 2). Consequently, changing total cellular levels of 
'O-GlcNAcylation does not appear to play a role in cellular distribution of FOXO4 as a reaction to oxidative stress. We now consider the role of O-GlcNAcylation and FOXO4 transcriptional regulation.

3.4. OGT enhances FOXO4 dependent transcriptional activity

The effect of O-GlcNAcylation on FOXO4 transcriptional activity was examined by reporter assays using FOXO-responsive promoters [19]. When co-transfected with FOXO4, OGT dramatically enhanced FOXO4 transcriptional activity on two different responsive element reporters, 6XDBE-LUC (Fig. 4A) and p27-DBE-LUC (Fig. 4C), and this was further increased with H₂O₂ treatment. This is also in agreement with previous reports showing H₂O₂ treatment led to increased FOXO4 transactivation activity in DBE-based luciferase assays [4,7]. OGT alone, or the JNK-mutant FOXO4-T447/451/454A had insignificant transcriptional activity (Fig. 4A&C). In contrast to OGT, the O-GlcNAcylase enzyme, NCOAT, decreased FOXO4 transcriptional activity (Fig. 4B), emphasizing the relevance of the role of O-GlcNAcylation in FOXO4 transcription.
4. DISCUSSION

Signaling pathways that decide the “survival vs die” fate of a cell often depend on post-translational modifications of cellular proteins. The response to oxidative stress involves members of the Forkhead family of transcription factors and in particular, FOXO4 can regulate anti-oxidant genes through phosphorylation, acetylation, and ubiquitination [4,8,21,22]. Here we demonstrate O-GlcNAcylation involvement in the oxidative stress response. Hydrogen peroxide caused increased association between OGT and FOXO4 proteins leading to O-GlcNAcylation of FOXO4 with enhanced transcriptional activity. Specifically, OGT and O-GlcNAc, promotes FOXO4-dependent transcriptional activity of p27\textsuperscript{Kip1}, causing cell cycle arrest [23], and enhanced cell survival (Fig 4C). Disruption of O-GlcNAcylation has also been shown to involve cell cycle arrest [24]. Conversely, the decrease in FOXO4 transcriptional activity by the O-GlcNAcase, (NCOAT, Fig.4B) could lead to a relaxing of cell cycle regulation and favor tumor growth.

Many previous studies implicate the elevation of O-GlcNAc as providing survival signals in response to various acute stress stimuli [15,25,26]. Specifically, O-GlcNAcylation of FOXO1 and FOXO3 proteins occur in response to nutrition and stress stimuli [10,11]. However, we did not detect O-GlcNAc modified FOXO1 (Fig. 1B) and FOXO3 (data not shown) in HEK293 cells under heat stress conditions. Housley et al [10] showed there are at least five potential O-GlcNAcylation sites on FOXO1 protein. Interestingly, H\textsubscript{2}O\textsubscript{2} caused relatively larger increases in O-GlcNAcylation than decreases in phosphorylation of FOXO4 (Ser193) (Fig. 2B), suggesting multiple O-GlcNAc sites. However, since we (Fig. 3, panels 2&4), and others [10] now show that O-GlcNAcylated
Fig. 4. OGT enhances FOXO4-dependent transcriptional activity. HEK293 cells were transfected with the FOXO responsive 6XDBE-Luciferase plasmid together with, (A) FOXO4, FOXO4-T447/451/454A mutant and OGT plasmids, or (B) FOXO4 and NCOAT plasmids, and treated with 200 µM H₂O₂ for 60 min. (C) Similar to Fig. 4A using p27-DBE-Luciferase. Data expressed as RLU of 6XDBE-Luciferase or p27-DBE-Luciferase divided by Renilla luciferase control, from three experiments shown as mean ± S.E. of triplicates. *P<0.05 vs. control.
FOXO proteins does not influence their AKT-dependent cellular redistribution, regulation of FOXO4 under oxidative stress may occur through its direct association with OGT, the exact mechanism yet to be determined.

In summary, our data describe a mechanism by which \( O\)-GlcNAc provides cell survival signaling in response to acute oxidative stress. OGT enhances FOXO4 transcriptional activity upon oxidative stress, causing the up-regulation of FOXO4 dependent target genes, such as cell cycle arrest and cell longevity genes, thereby promoting oxidative resistance.
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SUMMARY

Post-translational $O$-GlcNAc modification is involved in stress resistance and cell survival (Guinez et al., 2004; Zachara and Hart, 2004; Zachara et al., 2004). The current studies demonstrate that two evolutionary conserved pathways, the PI3 kinase and MAPK pathways, are also regulated by $O$-GlcNAc (Ho et al.). Upon acute oxidative stress, OGT can negatively modulate the JNK1 death signal and positively regulate the FOXO4 survival signal to promote protection for cells. The proposed working system is schemed as Figure (4).

**OGT modulates JNK1 to resist acute oxidative stress**

JNK, together with p38-MAPK, ERK1/ERK2 and ERK5, belongs to the evolutionarily conserved MAPK family of signaling proteins. Oxidative stress and mitogenic factors are able to activate MAPK phosphorylation signal transduction through cell-surface receptors, MAPKKK, MAPKK and MAPK, and their target transcription factors controlling gene expression. Among these MAPKs, JNK and p38-MAPK function mainly in stress dependent cell death (Derijard et al., 1994; Kyriakis et al., 1994; Raingeaud et al., 1995; Verheij et al., 1996). On the other hand, the $O$-GlcNAc activator, glucosamine, has been shown to provide benefits in several clinical disorders through MAPK signaling (Bissett, 2006). Cheung et al also showed that OGT directly interacts with activated p38-MAPK through its C-terminus and thus regulates $O$-GlcNAcylation of neurofilament H during glucose deprivation (Cheung and Hart, 2008). Furthermore,
Figure 1. The working model of $O$-GlcNAc on JNK1 and FOXO4 proteins function. OGT can positively regulate the FOXO4 survival signal (1) and negatively regulate the JNK1 death signal (2) to protect the cell from acute oxidative stress.
evidence indicates that $O$-GlcNAcylation has an inverse role with phosphorylation on many cellular proteins. Since MAPK is involved in the pathogenesis of many oxidative stress-mediated diseases and $O$-GlcNAcylation has benefit on stress resistance, it is both interesting and important to understand the role of $O$-GlcNAcylation on MAPK signaling.

In order to directly investigate the roles of OGT and NCOAT under oxidative stress, we established EGFP-tagged OGT and NCOAT stable HEK 293 cells. We showed that the OGT-overexpression of stable cells is more resistant to acute oxidative stress than control, and NCOAT-overexpressed stable cells in time and dose dependent manners. In addition, acute oxidative stress induced an association between OGT and JNK1 but not in ERK1 or p38-MAPK. OGT is also able to $O$-GlcNAcylate JNK1 and reduce its phosphorylation, suggesting that JNK1 $O$-GlcNAcylation is specific under oxidative stress. Altogether, these data provide a novel mechanism to link $O$-GlcNAcylation to oxidative stress resistance.

**$O$-GlcNAcylation enhances FOXO4 transcriptional regulation in response to stress**

FOXO transcription factors are able to modulate the expression of genes involved in stress dependent DNA damage repair process, cell cycle arrest and other cellular functions(Acilli and Arden, 2004; Huang and Tindall, 2007). Post-transcriptional modification plays an important role in regulation of cellular shuttling and activity of FOXO transcription factors. For example, PI3 kinase signaling can negatively regulate FOXO protein activity by direct AKT-dependent phosphorylation, causing their nuclear exclusion(Brownawell *et al.*, 2001). In contrast, Mdm2 causes FOXO4 nuclear
localization through mono-ubiquitination (Brenkman et al., 2008). Furthermore, oxidative stress causes SIRT1 to deacetylate FOXO proteins and modulates their activity (Brunet et al., 2004; van der Horst et al., 2004). More recently, researchers also found that FOXO1 is O-GlcNAcylated in response to glucose concentration (Housley et al., 2008; Kuo et al., 2008). However, the details of O-GlcNAcylation of FOXO proteins under oxidative stress have not been well documented.

In order to investigate the relationship between O-GlcNAcylation and FOXO transcription factors upon stress, we performed anti-O-GlcNAc immunoprecipitation in HepG2 cells and HEK293 cells (Ho et al.). Treatment of both cells with hydrogen peroxide or heat shock stress resulted in a dramatic increase in O-GlcNAc-modified FOXO4 levels indicating stress mediated FOXO4 O-GlcNAcylation. Further co-immunoprecipitation assays suggested that stress induced the physical association of OGT-FOXO4, principally through C-terminal of OGT. As a transcription factor, FOXO4 function is regulated at least in cellular localization and transcriptional activity. However, immunofluorescence data showed that changing total cellular levels of O-GlcNAcylation does not appear to play a role in FOXO4 cellular distribution. On the other hand, FOXO-responsive promoter reporter assays suggested that O-GlcNAcylation increased FOXO4 transcriptional activity. Specifically, OGT and O-GlcNAc, promote FOXO4-dependent transcriptional activity of p27kip1, causing cell cycle arrest and enhanced cell survival.

For future studies, mapping O-GlcNAcylation site(s) on FOXO4 and JNK1 protein would provide more detail for basic study and drug applications. Traditional mass spectrometry methods are not easily able to effectively identify protein O-GlcNAcylation site(s) due to its instability. The combination of chemical/enzymatic tagging,
photochemical cleavage, and electron transfer dissociation (ETD) mass spectrometry have been reported to efficiently identify $O$-GlcNAcylation sites (Wang et al., 2009b). The methods might be useful for identifying the $O$-GlcNAcylation site(s) of JNK1 and FOXO4 in the future studies.
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