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Investigation of the effects of increased levels of O-GlcNAc protein modification on protein kinase C and Akt

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Investigation of the Effects of Increased Levels of O-GlcNAc Protein Modification on Protein Kinase C and Akt

by

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Investigation of the Effects of Increased Levels of O-GlcNAc Protein Modification on Protein Kinase C and Akt

Jason Aaron Matthews

ABSTRACT

O-linked N-acetylglucosamine (O-GlcNAc) is an abundant and ubiquitous post-translational modification that has been shown to play a role in regulating a variety of intracellular processes. The pathway responsible for generating the O-GlcNAc modification, the hexosamine biosynthetic pathway (HBP), has also been shown to affect the activity and translocation of certain protein kinase C (PKC) isoforms. To investigate if the effects of HBP flux on PKC translocation observed by others is related to the O-GlcNAc modification, O-GlcNAc levels in human astroglial cells were elevated using four separate O-GlcNAc modulating agents followed by analysis of cytosol and membrane concentrations of PKC-ε, -α, -βII, and -ι. Of the four PKC isoforms analyzed, PKC-ε showed a significant reduction in its membrane associated levels in response to all agents tested whereas PKC-α showed reductions in response to only two agents.

Investigation of the mechanism for the reductions in membrane associated PKC-ε and -α indicate that the increased O-GlcNAc levels did not disrupt the activation of these isoforms or their ability to translocate to the plasma membrane. Furthermore, results indicate that these reductions are not due to a disruption in the Hsp70 mediated recycling of the isoforms. It was found; however, that increased O-GlcNAc levels resulted in increased degradation of PKC-ε suggesting that the decreases in membrane associated
PKC-ε may be a result of increased phosphatase or protease activity. Additional studies revealed that decreases in membrane bound PKC-α and PKC-ε, both of which act as anti-apoptotic enzymes, correlated with an increase in poly-(ADP-ribose) polymerase (PARP) cleavage – a well characterized hallmark of apoptosis.

In addition to PKC, the effects of increased O-GlcNAc levels on a related kinase, Akt, were also examined. Initial investigation of the effects of increased O-GlcNAc modification of Akt activation using glucosamine or streptozotocin revealed a relatively large, short-term increase in Akt phosphorylation in response to these treatments. However, further analysis with other O-GlcNAc modulators indicated that this activation was not related to O-GlcNAc protein modification. Furthermore, this activation does not appear to be related to any hyperosmotic effects associated with the treatment conditions, nor does it appear to be related to oxidative stress. Therefore, further investigation is needed to characterize the novel pathway responsible for Akt activation following glucosamine or streptozotocin treatment.
Chapter 1

O-GlcNAc protein modification

1.0 Introduction

Protein monoglycosylation occurs when N-acetylglucosamine (GlcNAc) from uridine diphosphate-GlcNAc (UDP-GlcNAc) is enzymatically transferred to nuclear and cytoplasmic proteins on serine or threonine hydroxyl groups forming β-O-linked N-acetylglucosamine moieties (O-GlcNAc). Unlike the more familiar multi-component glycosylation that occurs in the endoplasmic reticulum and Golgi, this single sugar addition occurs in the cytosol and is reversible. The O-GlcNAc residue is added to proteins by a specific UDP-GlcNAc: polypeptide O-β-N-acetylglucosaminyl transferase (OGT) [1-4] and removed by a N-acetyl-β-D-glucosaminidase (O-GlcNAcase) [5-9].

The UDP-GlcNAc used to form O-GlcNAc is generated via the hexosamine biosynthetic pathway (HBP) (Figure 1.1). In the HBP, fructose-6-phosphate is converted to N-acetylglucosamine-6-phosphate by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) [10,11]. Subsequent steps ultimately produce UDP-GlcNAc, the end product of the pathway (Figure 1.2).

1.1 O-GlcNAc Transferase

OGT is a highly conserved enzyme found in all metazoans studied from *Caenorhabditis elegans* to humans, including plants. In liver, kidney, and muscle cells,
Production of UDP-GlcNAc via the Hexosamine Biosynthetic Pathway

Figure 1.1 – Production of UDP-GlcNAc via the Hexosamine Biosynthetic Pathway

EC 2.3.1.4 glucosamine-phosphate N-acetyltransferase
EC 2.3.1.157 glucosamine-1-phosphate N-acetyltransferase
EC 2.6.1.16 glutamine—fructose-6-phosphate transaminase (isomerizing)
EC 2.7.7.23 UDP-N-acetylglucosamine diphosphorylase
EC 3.5.1.25 N-acetylglucosamine-6-phosphate deacylase
EC 3.5.99.6 glucosamine-6-phosphate deiminase
EC 5.4.2.3 phosphoacetylglucosamine mutase
EC 5.4.2.10 phosphoglucomannose mutase
O-GlcNAc Protein Modification via the Hexosamine Biosynthetic Pathway

Figure 1.2 – O-GlcNAc Protein Modification via the Hexosamine Biosynthetic Pathway [55]
this enzyme exists as a heterotrimer consisting of two 110kDa subunits and one 78kDa subunit [12]. In all other tissue analyzed, the 78kDa subunit is not expressed and the enzyme is comprised of three 110kDa subunits [14]. The 110kDa OGT subunit has a $K_m$ for UDP-GlcNAc of 0.545$\mu$M [12] and is inhibited by the resulting UDP product [13]. The active site for OGT has been shown to be in the 110kDa subunit [14,15]. Southern blot analysis of the OGT gene has shown it to be highly conserved from nematodes to man [14]. The OGT gene is located on the X-chromosome and is necessary for embryonic stem cell viability [16,17] and mice with the OGT gene knocked out die early in embryonic development [16] suggesting an important role for OGT in development.

The N-terminal domain of the 110kDa subunit contains 9-12 tetratricopeptide repeats (TPRs) [14,15]. The TPR domain is important for trimerization and stability of OGT [1]. OGT is both tyrosine phosphorylated and modified with O-GlcNAc [1,14]. No simple consensus motif on OGT substrates has been identified as the target for O-GlcNAc modification. Certain proteins, such as Ataxin-10 (the protein implicated in spinocerebellar ataxia) [18] and protein phosphatase-1$\beta$ and $\gamma$ have been shown to interact with OGT. OGT is ubiquitous but cytosolic OGT activity is 10 times greater in brain tissue than in muscle, adipose, heart, and liver tissue [3].

**1.2 O-GlcNAcase**

O-GlcNAcase, originally called hexosaminidase C, was first purified and characterized from rat spleen [8]. Like OGT, O-GlcNAcase is ubiquitous but is more highly expressed in brain tissue, placenta, and pancreas than in other tissues [6]. O-GlcNAcase is expressed in two forms, a 130kDa form and a splice variant with a molecular weight of 75kDa [6,19,20]. Evidence suggests that the 75kDa form is mostly
localized in the nucleus [20] whereas the 130kDa form is predominately cytoplasmic [6]. Unlike the 130kDa form, the 75kDa variant has no O-GlcNAcase activity and its role is unknown [9]. Unlike other hexosaminidases, O-GlcNAcase is found in the cytosol and nucleus as opposed to the lysosome, has a concomitantly more neutral optimal pH of 6.4 [6,8], and shows no other glycosidase activity [6,8]. O-GlcNAcase selectively removes GlcNAc, but not GalNAc, from glycopeptides [6,9]. Very little is known about the regulation of this enzyme and is an area for future study.

1.3 O-GlcNAc modification is reciprocal to O-phosphorylation

Studies investigating the possible relationship between O-GlcNAc and O-phosphate have demonstrated an inverse relationship (Figure 1.3). Treatment of cells with phosphatase inhibitors have led to decreased levels of O-GlcNAc [21,22] whereas cellular treatment with kinase inhibitors increases O-GlcNAc levels [21]. This reciprocal relationship has also been demonstrated on certain individual proteins. The C-terminal domain (CTD) of RNA polymerase II contains amino acid residues that are modified by both O-GlcNAc and O-phosphate [23,24]. Phosphorylation of the CTD blocks the ability of OGT to modify this domain and O-GlcNAc modification of the CTD blocks the ability of the CTD kinase to phosphorylate the region [25]. Additionally, the estrogen receptor-β [26] and SV-40 large T-antigen [27] have also been shown to be reciprocally modified with O-GlcNAc and phosphate. While this “yin-yang” relationship has been demonstrated on several proteins, others, such as c-Myc are capable of being modified with both O-GlcNAc and O-phosphate simultaneously [28] indicating that the “yin-yang” hypothesis is overly simplistic. Further evidence of this yin-yang relationship was demonstrated when OGT and protein phosphatase 1-β and -γ were shown to exist in
Hypothesized Yin-Yang Relationship Between O-GlcNAc and O-Phosphate

Figure 1.3 – Hypothesized Yin-yang relationship between O-GlcNAc and O-phosphate [56]
stable and active complex [29]. This complex was capable to dephosphorylating a synthetic peptide and then modifying the dephosphorylated residue with O-GlcNAc [29].

1.4 O-GlcNAc in cellular regulation

1.4.1 Protein regulation by O-GlcNAc

The O-GlcNAc protein modification has been demonstrated to function in the regulation of important signal transduction enzymes. In endothelial cells, increases in O-GlcNAc protein modification resulted in both the direct modification and reduced activity of phosphoinositide 3'-OH-kinase (PI 3-K) [30]. Also, increases in cellular O-GlcNAc modification have also been demonstrated to increase p42/44 and p38 MAPK activities and these increases are associated with activation of upstream MAPK kinases [31]. The activation of endothelial nitric oxide synthase (eNOS) in response to certain stimuli has also been shown to be impaired by increases in O-GlcNAc modification. Federici et al. [30] showed that the insulin-stimulated activation of eNOS was impaired as well as its ability to undergo phosphorylation in endothelial cells under conditions of increased O-GlcNAc modification [30]. Also, the activation of eNOS in the diabetic penis was shown to be impaired in response to fluid shear stress stimuli and vascular endothelial growth factor signaling and this impairment was a result of the modification of Ser-1177 by O-GlcNAc [32,32]. Additionally, the direct O-GlcNAc modification of the transcription factor Sp1 has been shown to inhibit its transcriptional capability [33].

1.4.2 Regulation of nuclear transport

The O-GlcNAc modification has been implicated in the regulation of nuclear transport. The nuclear pore proteins that are responsible for the active transport of protein in and out of the nucleus are highly enriched in O-GlcNAc [34] [35,36]. Also, the nucleus
contains a very high concentration of O-GlcNAc modified proteins [34]. Recently, the alpha4 phosphoprotein and Sp1, both of which contain O-GlcNAc moieties, showed a decreased translocation to the nucleus in response to decreases cellular O-GlcNAc levels [37].

1.4.3 Regulation of protein-protein interactions

The O-GlcNAc modification has been found on protein domains important for protein-protein interactions. Mutation of the O-GlcNAc modification site on the transcription factor Sp1 blocked its interactions with the Drosophila TAF110 protein and other Sp1 proteins [38]. The O-GlcNAc modification has also been shown to mediate interactions between Sp1 and a p62 glycoprotein [39]. Certain cytoskeletal proteins such as keratins 8, 13, and 18 [40-42], neurofilament proteins [8,43] have been demonstrated to contain O-GlcNAc modification sites in regions critical for protein-protein interactions. Other proteins such as the adenovirus fiber proteins 2 and 5 [44] and synapsin I [45] have sites shown to be modified with O-GlcNAc that are believed to mediate its interactions with other proteins.

1.4.4 Regulation of protein degradation

Proteins enriched with Pro, Glu, Ser, and Thr (PEST) sequences have been shown to be targeted for rapid degradation [46,47] following phosphorylation of these sequences. Research has shown that proteins that have high PEST sequences are also modified with O-GlcNAc [26,48,49] and this finding has led to the hypothesis that O-GlcNAc modification prevents phosphorylation of PEST sequences and thus prevents degradation of that protein. The cellular glycoprotein p67 has been shown to be deglycosylated and rapidly degraded under conditions of serum starvation and heme
depletion [50]. Also, the transcription factor Sp-1 has been shown to undergo proteosome dependent degradation when its level of O-GlcNAc modification is decreased [51]. Estrogen receptor-β is modified by O-GlcNAc on Ser19 [26] and when this residue was mutated to Ala16 in order to prevent its modification, its turnover rate was reduced; however, when Ser16 was mutated to Asp16 to mimic phosphorylation, its turnover rate was increased [52].

1.4.5 Regulation of stress response and cell cycle regulation

A variety of cellular stress including UVB irradiation and thermal stress has been shown to increase intracellular O-GlcNAc levels [53] and, conversely, decreases in O-GlcNAc levels have been shown to reduce the thermotolerance of mouse fibroblast cells [53]. Also, increases in O-GlcNAc have been shown to elevate Hsp-70 expression [53], a protein known to stabilize proteins and thus protect cells from thermal stress.

Increases in O-GlcNAc levels disrupt the progression of HeLa cells through the cell cycle by delaying G2/M progression [54]. Also, overexpression of O-GlcNAcase or OGT has been shown to disrupt mitotic phosphorylation and the timed expression of cyclin proteins [54]. Finally, increased OGT levels disrupted cytokinesis and resulted in aneuploidy [54].

1.5 O-GlcNAc and disease

1.5.1 O-GlcNAc and Diabetes

A large body of recent research has demonstrated a link between the O-GlcNAc modification and diabetes [55,56]. Increased levels of O-GlcNAc in adipocytes have been shown to result in decreased insulin stimulated glucose uptake [57]. Overexpression of OGT in mice resulted in insulin [58], and insulin resistance in muscle
cells due to the overexpression of Glut1 has been linked to increased levels of O-GlcNAc [59]. Analysis of the insulin signaling pathway has revealed many proteins associated with this pathway to be modified with O-GlcNAc suggesting an important regulatory role [30,60-62]. Phosphorylation of the insulin receptor substrate 1 and 2 (IRS1 and IRS2) is the first step in the insulin-signaling pathway after activation of the receptor. Cellular treatment with glucosamine, a compound shown to increase O-GlcNAc modification levels, results in a reduction of insulin-stimulated IRS1 tyrosine phosphorylation and a subsequent reduction downstream signaling events and also in modification of IRS-1 and IRS-2 with O-GlcNAc [60]. Recently, mass spectrometry analysis of IRS-1 has reveal Ser-1036 at the carboxyl-terminus to be the site of O-GlcNAc modification [62]. Pharmacological induced O-GlcNAc increases have resulted in the disrupted activation of the insulin signaling kinase, Akt, in response to insulin stimulation [57,61,63]. Park et al. [61] showed that increased O-GlcNAc levels in adipocytes reduced glucose uptake and GLUT4 translocation [61].

1.5.2 O-GlcNAc and Neurodegenerative disease

Many neuronal cytoskeletal proteins have been shown to be modified with O-GlcNAc [64], in particular the Tau and β-amyloid precursor protein. Tau, a microtubule binding protein associated in the pathology of Alzheimer’s disease, is both phosphorylated and extensively O-GlcNAc modified in normal brain tissue [64], and hyperphosphorylated tau is found in the aggregates of neurofibrillary tangles linked with Alzheimer’s. Together these discoveries have lead to the hypothesis that decreasing O-GlcNAc levels in the brain leads to the abnormal phosphorylation of tau [65]. Thus, O-GlcNAc may have a protective effect in the brain. Beta amyloid precursor protein (APP),
neurofilaments, and many synaptic vesicle proteins are also extensively modified with O-GlcNAc [49,66,67]. Further evidence suggesting a link between the O-GlcNAc modification and neurodegenerative diseases is the fact that both OGT and O-GlcNAcase map to loci linked to neurodegenerative diseases; the locus for OGT is associated with Parkinson’s disease, while the locus for O-GlcNAcase is linked with late onset Alzheimer’s disease.

1.5.3 O-GlcNAc and cancer

Although not yet extensively investigated, preliminary data indicates a relationship between increases in O-GlcNAc modification and breast cancer. Slawson et al. [68] demonstrated a decrease in total O-GlcNAc levels and a significant increase in O-GlcNAcase activity with alterations in the pattern of modified proteins in primary human breast carcinomas [68]. This data suggests that there is a disruption in the regulation of the O-GlcNAc modification as cells progress from normal to malignant possibly aiding the malignant phenotype [68].

1.6 Pharmacological modulation of O-GlcNAc levels

1.6.1 Glucosamine increases intracellular O-GlcNAc levels

Glucosamine is a compound known to enter the hexosamine biosynthetic pathway (HBP) downstream of the L-glutamine:L-fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme in the pathway (Figure 1.4 and 1.5). Since GFAT is regulated by negative feedback by its downstream product UDP-GlcNAc [11,69], the entry of glucosamine downstream of GFAT allows an increase in UDP-GlcNAc and ultimately increases in O-GlcNAc protein modification [70]. In addition to increasing
Structures of O-GlcNAc Increasing Agents

Glucosamine (GlcN)

O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc)

Streptozotocin (STZ)

1,2-dideoxy-2'-propyl-α-D-glucopyranos-2-[2,1-d]-Δ2' – thiazoline (NAGBT)

Figure 1.4 – Structures of O-GlcNAc Increasing Agents
Points of Action for Glucosamine, STZ, PUGNAc, and NAGBT

Figure 1.5 – Points of Action for Glucosamine, STZ, PUGNAc, and NAGBT [55]
UDP-GlcNAc levels, glucosamine treatment has also been demonstrated to have other cellular effects such as altered endoplasmic reticulum function [71] or calcium entry [72].

In addition to increasing O-GlcNAc levels, glucosamine treatment has been shown to have a variety of other effects on cells. Glucosamine treatment has been shown to cause depletions of intracellular ATP content, possible through the enhanced conversion of glucosamine to glucosamine-6-phosphate [73,74]. Also, treatment of macrophages with glucosamine was shown to inhibit Ca\(^{2+}\) influx across the plasma membrane that was unrelated to ATP depletion [72]. Additionally, the increased flux through the hexosamine biosynthetic pathway as a result of glucosamine treatment has been shown to lead to increased H\(_2\)O\(_2\) levels and increased oxidative stress in pancreatic \(\beta\)-cell [75]. This increased oxidative stress was determined to not be related to increases in the O-GlcNAc modification [75]. Finally, glucosamine has been shown to result in disruption of ER homeostasis that interferes with normal protein folding leading to an accumulation of misfolded or unfolded proteins [71,76], a condition known as ER stress [77].

1.6.2 STZ increases intracellular O-GlcNAc levels

Streptozotocin (STZ) is an antibiotic produced by the bacterium *Streptomyces achromogenes*. This compound is derived from glucose and 1-methyl-1-nitrosourea [61] forming an N-acetyl-glucosamine analog with an N-methyl-N-nitrosourea group linked to the C-2 carbon on the sugar (Figure 1.4). Over the last three decades, STZ has been used as a diabetic agent due to its ability to acutely induce pancreatic \(\beta\) cell death [78]. STZ has the ability to release nitric oxide as well as donate methyl groups to nucleotides and DNA [79-81] although recently its ability to release NO has been brought
into question [82]. STZ has also been demonstrated to be an inhibitor or O-GlcNAcase [5,83-85] and is believed to lead to elevated intracellular O-GlcNAc levels via this inhibition (Figure 1.5). Recently, STZ has been shown to inhibit O-GlcNAcase via the production of a transition state analog [86]. O-GlcNAcase converts STZ to a compound that closely resembles the natural ligand transition state only more energetically stable. The resulting analog is catalyzed to completion very slowly thus out competing the normal GlcNAc substrate for the enzyme’s active site [86].

1.6.3 PUGNAc increases intracellular O-GlcNAc levels

PUGNAc (O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate) was originally shown to be a potent \( \beta \)-N-acetylglucosaminidase inhibitor of a variety of different \( \beta \)-hexosaminidases [87] (Figure 1.4). It has also been demonstrated to be a highly potent inhibitor of O-GlcNAcase [8] and is effective at increasing intracellular O-GlcNAc levels without the cytotoxic effects associated with prolonged STZ treatment [7] (Figure 1.5). Recent studies have demonstrated that, although it is a potent O-GlcNAcase inhibitor, PUGNAc inhibits other \( \beta \)-hexosaminidases with similar K\(_i\) values [82] (Table 1.1).

1.6.3 NAGBT increases intracellular O-GlcNAc levels

NAGBT (1,2-dideoxy-2'-propyl-\( \alpha \)-D-glucopyranoso-[2,1-d]-\( \Delta \) 2'-thiazoine) is a recently synthesized compound generated to function as an effective O-GlcNAcase inhibitor [82] (Figure 1.4 and 1.5). Although not inhibiting O-GlcNAcase as potently as PUGNAc, it is much more selective for O-GlcNAcase over other \( \beta \)-hexosaminidases [82] (Table 1.1). It has also been shown to be effective at increasing intracellular O-GlcNAc modified proteins in African monkey kidney cells [82]. Due to its recent
characterization, no studies have been published to date using NAGBT to study O-GlcNAc effects and, therefore, any other effects that this compound may have in vitro are unknown.

Inhibition constants and selectivity of inhibitors for both O-GlcNAcase and β-hexosaminidase

<table>
<thead>
<tr>
<th>Compound</th>
<th>O-GlcNAcase $K_I$ µM</th>
<th>β-Hexosaminidase $K_I$ µM</th>
<th>Selectivity ratio ($\beta$-Hexosaminidase $K_I/O$-GlcNAcase $K_I$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc</td>
<td>1500</td>
<td>1200</td>
<td>0.8</td>
</tr>
<tr>
<td>STZ</td>
<td>1500</td>
<td>47,000</td>
<td>31</td>
</tr>
<tr>
<td>PUGNAc</td>
<td>0.046</td>
<td>0.036</td>
<td>0.8</td>
</tr>
<tr>
<td>NAGBT</td>
<td>0.23</td>
<td>340</td>
<td>1500</td>
</tr>
</tbody>
</table>

Table 1.1 – Inhibition constants for various O-GlcNAcase inhibitors [82]
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Chapter 2

Protein kinase C

2.0 Introduction

Protein kinase C (PKC) is a family of serine-threonine kinases that play critical roles in the regulation of cell growth, differentiation, and apoptosis [1,2]. There are 12 known PKC isoforms that are divided into three groups based upon their means of activation. The conventional PKC isoforms (cPKC) consist of PKC-α, βI, βII, and γ. These isoforms can be activated by Ca\(^{2+}\), 1,2-diacylglycerol (DAG) or phorbol 12-myristate 13-acetate (PMA), and phosphatidylserine (PS). The novel PKC isoforms (nPKC) consist of PKC-δ, ε, η, μ, and θ. These forms lack a Ca\(^{2+}\) binding domain but can still be activated by DAG, PS, and unsaturated fatty acids. Finally, the atypical PKC isoforms (aPKC) are PKC-ζ and ι (and its mouse homologue PKC-λ). These PKCs are only activated by PS, phosphatidylinositides and unsaturated fatty acids. Also, the more recently discovered PKC-μ (also called PKD) [3,4], is activated by phorbol esters [5] but, unlike other isoforms, contains a pleckstrin homology domain similar to Akt (Table 2.1).

Whereas PKC-α, βI, βII, δ, ε, and ζ have been shown to be ubiquitous [6-8], other PKC isoforms demonstrate distinct tissue localization. PKC-γ is predominately found in the tissues of the central nervous system [7,9,10], whereas PKC-η is expressed
<table>
<thead>
<tr>
<th>PKC isoform</th>
<th>M&lt;sub&gt;w&lt;/sub&gt; (kDa)</th>
<th>Number of amino acids</th>
<th>Activators</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPKC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>76.8</td>
<td>672</td>
<td>PS, Ca&lt;sup&gt;2+&lt;/sup&gt;, DAG, FA, 1,25-D&lt;sub&gt;3&lt;/sub&gt;, PMA</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>β I</td>
<td>76.8</td>
<td>671</td>
<td>PS, Ca&lt;sup&gt;2+&lt;/sup&gt;, DAG, FA, PMA</td>
<td>most tissues</td>
</tr>
<tr>
<td>β II</td>
<td>76.9</td>
<td>673</td>
<td>PS, Ca&lt;sup&gt;2+&lt;/sup&gt;, DAG, FA, PMA</td>
<td>most tissues</td>
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<tr>
<td>γ</td>
<td>77.9</td>
<td>697</td>
<td>PS, Ca&lt;sup&gt;2+&lt;/sup&gt;, DAG, FA, 1,25-D&lt;sub&gt;3&lt;/sub&gt;, PMA</td>
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</tr>
<tr>
<td>nPKC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>77.5</td>
<td>674</td>
<td>PS, DAG, FA, PI, PMA</td>
<td>ubiquitous</td>
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<tr>
<td>ε</td>
<td>83.5</td>
<td>737</td>
<td>PS, DAG, PI, 1,25-D&lt;sub&gt;3&lt;/sub&gt;, PMA</td>
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<tr>
<td>η</td>
<td>78.0</td>
<td>683</td>
<td>PI, PMA</td>
<td>neural, epithelium</td>
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<tr>
<td>θ</td>
<td>81.6</td>
<td>707</td>
<td>PI, PMA</td>
<td>ovary, skeletal muscle, platelets</td>
</tr>
<tr>
<td>aPKC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ξ</td>
<td>67.7</td>
<td>592</td>
<td>PS, FA, PIP3, ceramide, PA</td>
<td>most tissues</td>
</tr>
<tr>
<td>ι/λ</td>
<td>67.2</td>
<td>586</td>
<td>unknown</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>PKC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>μ</td>
<td>115.0</td>
<td>912</td>
<td>unknown</td>
<td>unknown</td>
</tr>
</tbody>
</table>

**Table 2.1 – **Protein kinase C isoform data. PS – phosphatidylserine; DAG – diacylglycerol; FA – fatty acid; 1,25-D<sub>3</sub> - 1,25-Dihydroxycholecalciferol; PMA – phorbol 12-myristate 13-acetate; PI – phosphatidylinositol; PA – phosphatidic acid. [51]
largely in skin and lung [11]. PKC-θ is present in skeletal muscle and also in lung, spleen, skin, and brain [12] and PKC-μ is largely found in thymus and lung [13].

2.1 Protein kinase C structural domains

All of the PKC groups are composed of an NH₂-terminal regulatory region and a COOH-terminal catalytic region [14]. The cPKCs are composed of four conserved domains (C1-C4) separated by five variable domains (V1-V5). The C1, C2, V1, and V2 domains are located on the regulatory region whereas the C3, C4, V4, and V5 domain are located on the catalytic region. The V3 domain, also called the hinge region, connects the regulatory and catalytic regions (Figure 2.1)

The C1 domain is a small globular structure approximately 8kDa consisting of several cysteine-rich repeats [15]. These cysteine-rich repeats resemble zinc finger domains found in many DNA-binding proteins, although there is no evidence that any PKC isoforms bind DNA [15]. Evidence suggests that two Zn²⁺ atoms bind each Zn finger and coordinate with a histidine and three cysteine residues thus stabilizing a particular conformation [16]. This C1 domain is also essential for DAG and phorbol ester binding and also interacts with membrane lipids [17,18]. The binding site for these hydrophobic molecules is formed by two pulled-apart β sheets [15]. cPKCs and nPKCs have two C1 domains (C1A and C1B), but evidence suggests that only one of these participates in ligand binding [19]. aPKCs contain a nonfunctional C1 domain that lacks the key residues necessary for DAG or phorbol ester binding [19].

The C2 domain is a 12 kDa β-strand rich region required for Ca²⁺ binding. This domain contains several acidic residues thought to participate in this binding [20,21].
Protein kinase C Isoforms' Structural Domains

Figure 2.1 - Protein Kinase C isoforms' structural domains [14]
nPKCs lack the key residues in the C2 domain involved in Ca\(^{2+}\) binding [22], and aPKCs lack a C2 domain altogether [6].

The C3 domain contains the ATP binding site and the C4 domain is the substrate recognition site [23]. All of the PKC isoforms contain C3, V4, C4, and V5 domains that make up the catalytic domain [24]. In addition to these domains, PKCs have a pseudosubstrate sequence (amino acids 13-30) that precedes the C1 domain [25]. This region contains a motif resembling the consensus sequence found in the phosphorylation sites of PKC substrates; however, it cannot be phosphorylated [26]. In PKC’s inactive state, it interacts with the active site through Arg19 [27]. This arginine interacts with several acidic residues in the enzymes active site thus blocking the enzyme from becoming catalytically active [27].

2.2 Protein kinase C activation

2.2.1 Activation by phosphorylation

All PKC isoforms are initially transcribed as a single unphosphorylated polypeptide chain. Studies of PKC-βII show that the enzyme must undergo three phosphorylations before it is capable of activation by DAG [28]. These phosphorylation sites are conserved among the PKC isoforms [24] (Figure 2.2). Newly transcribed PKC-βII initially has an apparent molecular weight of 76kDa and is found in the detergent insoluble fraction on cells [28]. It undergoes phosphorylation on Thr500 by 3’-phosphoinositide-dependent kinase-1 (PDK-1) at a segment near the entrance of the active site referred to as the activation loop [29-32]. This phosphorylation does not affect its electrophoretic mobility on SDS-PAGE. It then is autophosphorylated at Thr641 at a region known as the turn motif altering its electrophoretic mobility and producing an
Protein Kinase C Phosphorylation Sites

Figure 2.2 – Protein Kinase C Phosphorylation Sites [21]
enzyme with an apparent molecular weight of 78kDa [28]. Finally, PKC is
autophosphorylated at Ser660 in a region called the hydrophobic motif giving it an
apparent molecular weight of 80kDa [28]. After this final phosphorylation, the enzyme is
released from the cytoskeleton into the cytosol [28] where it is capable of being activated
by various cofactors.

2.2.2 Activation by cofactors

Once the fully phosphorylated PKC is released into the cytosol and the enzyme
remains inactive due to its active site being occupied by its NH₂-terminal pseudosubstrate
[33]. This occupation blocks the active site and prevents enzymatic activity. Upon
increases in intracellular Ca²⁺, the C2 domain of the phosphorylated cPKCs binds this
free Ca²⁺. As the cPKC diffuses through the cell and contacts the membrane, the bound
Ca²⁺ forms a low-affinity interaction with anionic phospholipids in the membrane
[34,35]. Once at the membrane, PKC can then interact with membrane embedded
diacylglycerol via its C1 domain. This high-affinity interaction results in a
conformational change that removes the pseudosubstrate from the substrate-binding site
[33]. The nPKCs lack a C2 domain and, therefore, do not bind Ca²⁺. Consequentially,
their translocation rate is an order of magnitude less than the cPKCs. The aPKCs do not
bind Ca²⁺ or diacylglycerol and therefore do not translocate in response to certain stimuli
as do the cPKCs and nPKCs.

2.3 Protein kinase C regulation by anchoring proteins

The biological function and substrate specificity of PKC is due in large part to its
cellular localization [36]. Correct localization of active PKC positions it near the proper
substrates and regulators such as phosphatases. Proteins called RACKs (receptors for
activated C-kinase) have been shown to bind active PKC in an isoform specific manner and localize them to specific cellular sites [37]. RACKS also bind other signaling enzymes such as phospholipase Cγ (PLCγ) [38], and PKC substrates such as dynamin-1 [39] linking them to the PKC isoform. The binding of RACK to active PKC has also been demonstrated to enhance PKC catalytic activity [40]. Thus far, two RACKs have been identified. RACK1 selectively binds PKC-βII and εRACK (also called βCOP) binds PKC-ε. RACK 1 and εRACK contain seven repeats of the WD40 motif [41,42] that is known to be involved in protein-protein interactions. The binding of RACK1 to PKC-βII occurs at the C2 domain of PKC-βII [43].

2.4 Protein kinase C deactivation

The activated conformation of PKC is highly sensitive to dephosphorylation [44] and is largely mediated by protein phosphatase-1 and -2A [45]. Once dephosphorylated, PKC localizes to the detergent-insoluble fraction [46] of the cells where it ultimately is degraded by proteolysis [47,48]. In addition to undergoing proteolysis, dephosphorylated PKC can also be recycled back to the membrane [49]. The dephosphorylated turn motif of PKC provides a binding site for the molecular chaperone heat shock protein-70 (Hsp70) [49]. This binding stabilizes PKC and allows it to cycle back to the cytosolic PKC pool where it can undergo another activation cycle [49,50] (Figure 2.3).
Protein Kinase C Life Cycle

Figure 2.3 – Protein Kinase C life cycle [50]
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Chapter 3

Glial Cells

3.0 Introduction

The central nervous system (CNS) is composed of both neurons and glial cells. In the brain, glial cells are up to fifty times more abundant than neurons [1] and are known as the "supporting cells" of the nervous system. The four main functions of glial cells are to surround neurons and hold them in place, to supply nutrients and oxygen to neurons, to insulate one neuron from another, and to remove dead or injured neuron via phagocytosis [2-4]. There are two types of glial cells: macroglial and microglial. Macrogial cells can be further divided into Schwann cells, located in the peripheral nervous system (PNS), and oligodendrocytes, ependymal glia, and astrocytes of the CNS (Figure 3.1 and 3.2).

3.1 Microglia

Microglia are the smallest of all the glial cells. They are the primary immune effectors in the CNS and have distinct morphologies and staining characteristics from other glia and neurons [5,6]. Microglia have a comparable function to macrophages and serve as scavenger cells in the event of infection, inflammation, trauma, ischemia, and neurodegeneration in the CNS [6,7]. Microglia are believed to originate from monocytes that enter the brain during embryonic development and differentiate into brain resident microglia [4]. They are found throughout the brain but appear to be more densely located in the gray matter near blood vessels. In response to brain injury, microglia undergo a
Glial cells of the central nervous system

Figure 3.1 - Glial cells of the central nervous system. [39]
Types of Glial Cells

Astroglia

Microglia

Oligodendroglia

Schwann cell

Figure 3.2 - Types of glial cells [40]
change in morphology to an ameboid shape and are then able to migrate to the insult site where they can engulf foreign organisms or damaged cells by phagocytosis [8]. Activated microglia are also capable of releasing cytotoxic substances such as oxygen radicals, proteases, and proinflammatory cytokines [9,10].

3.2 Schwann Cells

Schwann cells ensheathe and myelinate most of the surface of all axons in peripheral nerves. Schwann cells originate from cells in the neural crest and can be divided into two types based on their morphology: myelin-forming and nonmyelin-forming [11]. Both types of Schwann cells ensheathe the neuronal axons although by different means. A single nonmyelinating Schwann cells can ensheathe several small diameter (<1µM) axons by forming small invaginations on its surface in which individual unmyelinated axons sit [12]. The roles of nonmyelinating Schwann cells have been poorly investigated but may play a role in the maintenance of unmyelinated axons [12]. Alternatively, the more well studied myelinating Schwann cells associate with only one large diameter (>1µM) axon and wrap around it numerous times to form the multilaminated and highly compacted myelin sheaths that underlie fast, saltatory conduction [12]. Myelinating Schwann cells influence the structure of the axons they ensheathe including axon diameter, axonal neurofilament spacing, and phosphorylation [13]. The gaps between the Schwann cell covered segments, called the Nodes of Ranvier, serve as important sites of ionic and other exchanges of the axon with the extracellular liquid.

3.3 Oligodendrocytes
Similar to the Schwann cells of the PNS, oligodendrocytes ensheath neuronal axons in the CNS. One oligodendrocyte may ensheath anywhere from 5 to 30 axons by forming either a loose wrapping around a group of unmyelinated axons or forming tight multilaminar myelin sheaths [5,14]. Similar to astrocytes and ependymal glia, oligodendrocytes arise from the ectoderm of the developing neuroepithelium [14].

The principle function of oligodendrocytes is to provide support to axons and to produce the myelin sheath that insulates axons. Myelin is 80% lipid and 20% protein and allows for the efficient conduction of action potentials down the axon [3]. Unlike Schwann cells, oligodendrocytes form segments of myelin sheaths of numerous neurons at once. Each process from a given oligodendrocyte can wrap itself around portions of a nearby axon forming layers of myelin [3] and becoming a segment of the axon's myelin sheath (Figure 3.3).

3.4 Ependymal Glia

Ependymal cells are epithelial cells that line the central cavities of the brain and the spinal cord [15]. They range in shape from squamous to columnar and in certain regions of the brain they possess cilia [15]. Ependymal cells form a relatively permeable barrier between the cerebrospinal fluid that fills those cavities and the tissue fluid that surrounds the cells of the CNS. In certain region of the brain ependymal cells possess cilia, the beating of which help to circulate the cerebrospinal fluid that cushions the brain [16]. Modified ependymal cells contribute to the formation of the choroids plexus which is a capillary knot that protrudes into a brain ventricle, and is involved in the synthesis of cerebrospinal fluid [17]. The cell bodies and nuclei of ependymal glia, however, are located primarily in the ependymal layer of the brain with their processes extending to
Figure 3.3 - Oligodendrocyte [41]
nearby blood vessels [18]. They can be distinguished from astrocytes based on their apparent growth-promoting properties in adult brain, their expression of both p75 and the estrogen receptor, and their ability to survive and proliferate in culture [19,20]. In adult mammals, the cerebral ventricles are normally lined by a layer of cuboidal and multiciliated ependymal cells [21,22]. These cells are at the interface between the brain parenchyma and the ventricular cavities and play an essential role in the propulsion of CSF through the ventricular system [16,23]. The coordinated beating of cilia in ependymal cells creates a current of CSF along the walls of the lateral ventricle; ependymal malfunction leads to disturbances of CSF flow and hydrocephaly [24-26]. It has also been suggested that ependymal cells filter brain molecules [27], insulate the brain from potentially harmful substances in the CSF [28], move cellular debris in the direction of bulk CSF flow, and optimize the dispersion of neural messengers in the CSF [29].

3.5 Astrocytes

Astrocytes, also called astroglial cells, are the most abundant types of glial cell. They are easily distinguishable from other types of glial cells during development due to their robust expression of the cytoskeletal proteins GFAP and vimentin [5]. Astrocytes are generally star shaped cells with numerous projections that anchor neurons to their blood supply and are found in both the white and gray matter of the brain. They ensheath regions of CNS neurons that are not covered by oligodendrocytes, such as the Nodes of Ranvier [2] and also encapsulate synaptic regions between neurons [2]. Both astrocytes and ependymal cells have processes that contact blood vessels and the pial surface of the CNS suggesting a role in trafficking metabolites and eliminating waste products to and
from neurons. Astrocytes are coupled together with gap junctions that allow the movement of ions and small molecules between them [30].

Astrocytes carry out a large number of functions in the CNS including modulation of synaptic function [31]. Astrocytes actively take up the neurotransmitter glutamate via specific transporters, thereby shortening the synaptic current and protecting postsynaptic cells from potentially excitotoxic effects [31]. They then convert the glutamate back to glutamine via glutamine synthetase and shuttle it back to neurons where it is converted back to glutamate [31] (Figure 3.4).

Astrocytes also regulate the neuronal microenvironment by controlling the extracellular pH and $K^+$ levels [32], and the supply of various metabolic substrates [2]. Astrocytes provide nourishment to neurons by receiving glucose from capillaries, breaking the glucose down into lactate, and releasing the lactate into the extracellular fluid surrounding the neurons [33]. The neurons receive the lactate from the extracellular fluid and transport it to their mitochondria to use as an energy source [33]. In this process astrocytes store a small amount of glycogen, which stays on reserve for times when the metabolic rate of neurons in the area is especially high [34]. In addition to regulating the neuronal microenvironment, astrocytes also affect neuronal development via the release of neurotrophic factors [35] and increase the number of mature, functional synapses on CNS neurons [36].

Finally, astrocytes function as the primary cell type responsible for the formation and maintenance of the blood brain barrier (BBB), the structure that limits the entry of blood-borne elements in the CNS [2]. It has been demonstrated that astrocyte ablation leads to the failure of the BBB to repair [37]. Additionally, astrocytes may regulate
vasoconstriction and vasodilation by producing substances such as arachidonic acid, whose metabolites are vasoactive.
Figure 3.4 - Glutamate-glutamine cycling between central astrocytes and neurons.

Abbreviations: α-KG, α-ketoglutarate; GS, glutamine synthetase; PAG, phosphate-activated glutaminase; SNAT1/2, system N/A amino acid transporter [38].
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Chapter 4

Effects of increased O-GlcNAc protein modification on Protein kinase C translocation

4.0 Introduction

The O-GlcNAc protein modification has been demonstrated to function in the regulation of important signal transduction enzymes. The activity of certain kinases, such as PI 3-K [1] and p42/44 and p38 MAPK [2] have been shown to be affected by increases in cellular O-GlcNAc protein modification (further discussed in section 1.5.1). In certain systems flux through the hexosamine biosynthetic pathway (HBP), the pathway responsible for producing the substrate for the O-GlcNAc transferase (OGT), has been shown to affect the activity of protein kinase C (PKC) [3-7]. Filippis [7] showed that increased flux through the HBP led to a 3-fold increase in overall PKC activity. Others have shown that flux through the HBP regulates the translocation of specific PKC isozymes [3,6]. Although PKC regulation has been linked to flux through the HBP, a direct connection to O-GlcNAc levels has not been investigated.

A good model system to investigate the relationship between PKC and O-GlcNAc is the brain. Brain tissue has been shown to express higher levels of PKC [8,9] and O-GlcNAcase [10] and have 10 times greater OGT activity [11] than most other tissues. Therefore we examined this relationship in a SV-40 transformed human glial (SVG) cell line that has previously been used as a human astroglial model system [12,13]. This
chapter discusses experiments done in order to investigate the effects of increasing O-GlcNAc protein modification on the translocation of several PKC isozymes. It also compares and contrasts the effects of four O-GlcNAc modulating agents on the rates and levels of O-GlcNAc increases in SVG cells.

4.1 Materials and methods

4.1.1 Materials

SVG cell line, eagle’s minimum essential medium (EMEM) and fetal bovine serum (FBS) were obtained from American Type Culture Collection (Rockville, MD). Penicillin/streptomycin was purchased from Fisher Scientific (Suwanee, GA). PKC-α (sc8393), PKC-βII (sc210), and PKC-ε (sc214) specific antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while PKC-ι (P20520) was obtained from Transduction Laboratories (San Diego, CA). CTD110.6 anti-O-GlcNAc antibody was a kind gift from Dr. Gerald Hart at Johns Hopkins University (Baltimore, MD) and is now available from Covance Research Products (Berkeley, CA). Goat anti-mouse-HRP and goat anti-rabbit-HRP secondary antibodies were from BioRad (Hercules, CA). D-glucosamine and streptozotocin (STZ) were purchased from Sigma (St. Louis, MO) and O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc) was from Carbogen (Aarau, Switzerland). 1,2-dideoxy-2'-propyl-α-D-glucopyranosyl-[2,1-d]-Δ2'-thiazoline (NAGBT) was a kind gift from Dr. David J. Vocadlo at Simon Fraser University (British Columbia, Canada). All other chemicals were purchased from Sigma and were of the purest grade available.

4.1.2 Cell culture
SVG cells were grown at 37°C in a 95% air and 5% CO₂ environment. Cells were cultured in T-150cm² flask to 90% confluency in EMEM (5.4mM glucose) supplemented with 10% FBS and 10U/mL penicillin and 10µg/mL streptomycin. Cell media was supplemented with 8mM glucosamine, 5mM STZ, 80µM PUGNAc, or 75µM NAGBT for the time periods indicated.

4.1.3 Fractionation of cytosolic and membrane proteins

To terminate experiments, flasks of cells were immediately placed on ice and washed with ice cold phosphate buffered saline (PBS). Cells were scraped into PBS and centrifuged a 2,000xg for 3 minutes to pellet cells. PBS was removed and cell pellet was resuspended in 550µl of ice-cold homogenization buffer (20mM Tris, 1mM EDTA, 100mM NaCl, 1mM dithiothreitol, 1mM PMSF, 1mM Na₃VO₄, 4µg/ml aprotinin, pH 7.4). Cells were lysed by sonication (Fisher Scientific Sonic Dismembrator F60) on ice with 2, 10-second pulses at 7 watts. Any whole cells or debris was pelleted by centrifuging at 1000xg for 5 minutes at 4°C. Supernatants were then centrifuged at 100,000xg for 1 hour at 4°C. The resulting supernatants were removed and labeled the cytosol fraction. Pellets were resuspended in homogenization buffer with 1.0% Triton X-100 by gentle agitation for 30 minutes followed by a brief 2-second sonication at 7 watts. Samples were centrifuged at 100,000xg for 30 minutes at 4°C. Supernatants were removed and labeled the membrane fractions. Protein concentrations of both fractions were determined using BioRad Protein Assay Dye Reagent using bovine serum albumin (BSA) as a standard.
4.1.4 Electrophoresis and Western blotting

Proteins from cytosol and membrane fractions were mixed with 0.3 volumes of 3x sample buffer (0.18M Tris-HCl pH 6.8, 6% sodium dodecyl sulfate (SDS), 30% glycerol, 0.025% Bromophenol Blue) and equal amounts of protein were loaded onto 8% SDS-polyacrylamide gels. Samples were electrophoresed for 1 hour 30 minutes at 15mAmps and then transferred to nitrocellulose membranes by electroblotting in 50mM Tris, 77mM glycine, and 20% methanol transfer buffer for one hour at 12 volts. For western blot analysis with PKC antibodies, membranes were blocked with 5% (w/v) non-fat dried milk in tris buffered saline (140mM NaCl, 2.7mM KCl, and 25mM Tris pH 8.0) with 0.05% Tween-20 (TBS-T). Membranes were then incubated overnight at 4°C in TBS-T with 3% milk containing the PKC antibody. PKC-α antibody was diluted 1:15,000, PKC-βII antibody was diluted 1:1,500, PKC-ι antibody was diluted 1:150, and PKC-ε was diluted 1:1000 for western blot analysis. Membranes were subsequently washed for 10 minutes (5 times) in TBS-T with 1% milk. Either goat anti rabbit-HRP (1:6000 dilution for PKC-βII and PKC-ε) or goat anti mouse-HRP (1:20,000 dilution for PKC-α and 1:5000 dilution for PKC-ι) in TBS-T 1% with milk was then incubated with the membranes. Membranes were washed again 10 minutes (6 times) in TBS-T with 1% milk and bands were detected by chemiluminescence according to the manufacture’s instructions (Pierce, Rockford, IL).

For analysis using CTD110.6 antibody, membranes were blocked in tris buffered saline with 0.3% Tween-20 (TBS-HT) for 1 hour then incubated overnight at 4°C in TBS-HT containing the CTD110.6 antibody (1:5000 dilution) [14]. Membranes were washed for 10 minutes (2 times) in tris buffered saline with 1% Triton X-100, 0.1% SDS,
0.25% deoxycholic acid (TBS-D) and (3 times) in TBS-HT. Goat anti rabbit Ig-M-HRP (1:15,000 dilution) in TBS-HT was then added to membranes. Membranes were washed as before and bands detected using chemiluminescence. Immunoblots were quantified using Scion Image 4.02 analysis program (Scion Corp., Frederick, MD).

4.1.5 Statistical Analysis

Data are given as ± standard error of the mean (S.E.M) for three to five experiments. Comparisons between means were performed using two-tailed Student’s t test for unpaired data and graphed using SigmaPlot 8.0™. Values with p<0.05 were considered significant.

4.2 Results

4.2.1 Glucosamine, STZ, PUGNAc, and NAGBT increase O-GlcNAc modification on proteins

In order to examine the effect of increasing O-GlcNAc modification on PKC translocation to the plasma membrane, an SV-40 transformed human glial cell line (SVG) was treated independently with three compounds at levels known to increase O-GlcNAc in other systems [15-17]. SVG cells were exposed to either 8mM glucosamine, 5mM STZ, 80µM PUGNAc, or 75µM NAGBT for 1, 3, 5 and 9 hours. In conjunction with these time points, four untreated (control) samples were also examined in order to determine O-GlcNAc and PKC basal levels. Each experiment was repeated between three and five times as indicated.

To confirm that these three compounds did indeed increase the intracellular O-GlcNAc modification in this system and to compare each one’s effect, we analyzed both the cytosolic and membrane fractions from treated and untreated SVG cells. The
fractions were analyzed by SDS-PAGE followed by immunoblotting with the CTD 110.6 antibody that specifically recognizes the O-GlcNAc modification [18]. Western blot analysis of cytosol and membrane fractions showed, as expected, a significant increase in O-GlcNAc levels but with quantitative differences between the compounds. Both cytosol and membrane fractions showed numerous labeled proteins with the most prominent increases on proteins of 125kDa, 96.3 kDa, 89.0 kDa, 66.0 kDa, and 35.1 kDa in the cytosol (Figure 4.1). Membrane fractions showed major increases in O-GlcNAc content on proteins between 134 kDa and 76.6 kDa and at 55.1 kDa (Figure 4.2). O-GlcNAc levels for all fractions were also quantified densitometrically by measuring the intensity of banding between 200 and 50 kDa (data not shown). Glucosamine treated samples showed maximum O-GlcNAc levels in both the cytosol and membrane fractions as early as one hour post treatment, increasing by ~43% in the cytosol and ~37% in the membrane fraction versus untreated samples. These levels remain nearly constant throughout the nine hour treatment period. STZ treated samples were at maximum O-GlcNAc levels as early as three hours after treatment in the cytosol (~51% increase) and five hours in the membrane fraction (~89% increase). Unlike glucosamine and STZ treatment, PUGNAc treatment continued to show increased O-GlcNAc throughout the time course. After nine hours of PUGNAc treatment, O-GlcNAc levels increased by ~121% in the cytosol and ~236% in the membrane. Densitometric analysis of these immunoblots revealed that 75µM NAGBT treatment increased overall O-GlcNAc levels on both cytosol and membrane associated proteins to approximately twice the levels seen following 8mM glucosamine or 5mM STZ treatment (Figure 4.1 and 4.2). A comparison of O-GlcNAc levels following 75µM NAGBT and 80µM PUGNAc treatment revealed interesting
A. Cytosol

Figure 4.1 - Effects of glucosamine, STZ, PUGNAc, and NAGBT treatments on O-GlcNAc modification of cytosolic proteins. SVG cells were treated with either 8mM glucosamine, 5mM STZ, 75µM NAGBT, or 80µM PUGNAc for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), or 9 hours (T=9). Untreated (control) samples were also prepared. Cells were fractionated into cytosol and membrane fractions and western blotted for O-GlcNAc using the CTD110.6 anti-O-GlcNAc antibody.
Figure 4.2 - Effects of glucosamine, STZ, PUGNAc, and NAGBT treatments on O-GlcNAc modification of membrane proteins. SVG cells were treated with either 8mM glucosamine, 5mM STZ, 75µM NAGBT, or 80µM PUGNAc for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), or 9 hours (T=9). Untreated (control) samples were also prepared. Cells were fractionated into cytosol and membrane fractions and western blotted for O-GlcNAc using the CTD110.6 anti-O-GlcNAc antibody.
similarities and differences. The effect of the compounds on cytosolic O-GlcNAc levels was very similar resulting in a 40% increase after one hour of treatment that increased to 147% after 9 hours of treatment. However, analysis of membrane fractions from cells treated with 75µM NAGBT revealed a much slower increase than in cells treated with 80µM PUGNAc (Figure 4.2). After 3 and 5 hours of NAGBT treatment, overall amounts of the protein modification on membrane proteins had increased by only about 15% whereas PUGNAc treatment over the same duration resulted in an overall 40% increase in the membrane fraction. By 9 hours of treatment, O-GlcNAc increases in membrane fractions produced by both NAGBT and PUGNAc were at nearly identical levels (50% and 54% respectively) (Figure 4.2).

4.2.2 Effects of glucosamine on PKC translocation

In order to determine the effect of increased O-GlcNAc on active, membrane bound PKC, membrane fractions from SVG cells treated with glucosamine, STZ, or PUGNAc were separated by SDS-PAGE, immunoblotted, and probed with antibodies against PKC-α, PKC-βII, PKC-ι, and PKC-ε. In glucosamine treated samples, PKC-βII membrane concentrations increased by 41%±13% (p<0.015) compared to untreated control cells one hour after glucosamine treatment. PKC-βII continued to increase in the membrane fraction showing a maximal 73%±8% (p<0.00005) increase after three hours of treatment and 55%±17% (p<0.005) after five hours (Figure 4.3). In contrast to increases in PKC-βII translocation, membrane associated PKC-ε levels decreased after incubation with glucosamine. PKC-ε levels began decreasing after five hours of treatment, and, after nine hours of incubation, PKC-ε levels in the membrane fraction had
decreased by 48%±15% (p<0.005) (Figure 4.3). In contrast to PKC-βII and PKC-ε, membrane bound concentrations of PKC-α and PKC-ι showed no significant change compared to control cells (Figure 4.3).

The concentration of these PKC isoforms was also measured in the cytosol fraction; however, no significant changes were observed (data not shown). This is probably due to the fact that in unstimulated cells, the PKC concentration in the cytosol is much greater than in the membrane. Therefore, upon activation, changes in membrane concentrations can be significant while the corresponding change in the cytosol may be much less observable [6].

4.2.3 Effects of streptozotocin on PKC translocation

Cells treated with STZ were analyzed similar to those treated with glucosamine. In contrast to glucosamine treatment, STZ treatment resulted in a decrease in membrane bound PKC-α (Figure 4.4) while not increasing PKC-βII translocation (Figure 4.4). PKC-α underwent a 78%±10% (p<0.005) decrease after nine hours of incubation. Similar to glucosamine treatment, STZ treatment did not result in any significant change in PKC-ι (Figure 4.4) while producing a decrease in membrane bound PKC-ε. PKC-ε showed a 42%±7% (p<0.04) decrease after five hours of incubation and continued to decrease by an average of 87%±6% (p<0.0005) nine hours after addition of STZ (Figure 4.4). Also of note is that, although STZ has been shown to be toxic in pancreatic β-cells [19,20], it has been effectively used to increase O-GlcNAc levels in other cell lines without a decrease in cell viability [21]. During our nine hour STZ treatment period, we
Figure 4.3 - Effects of Glucosamine on PKC isoforms in membrane fractions.
SVG cells were treated with 8mM glucosamine for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Membrane fractions were treated with isoform specific anti PKC antibodies. Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for membrane fractions probed with anti PKC-βII, PKC-ι, PKC-α, and PKC-ε antibodies are shown (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01.
Figure 4.4 - Effects of STZ on PKC isoforms in membrane fractions.
SVG cells were treated with 5mM STZ for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18μg) were separated by SDS-PAGE, and transferred. Membrane fractions were treated with isoform specific anti PKC antibodies. Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for membrane fractions probed with anti PKC-βII, PKC-ι, PKC-α, and PKC-ε antibodies are shown (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01
observed no changes in cell morphology or total protein levels or loss of cell viability (data not shown).

4.2.4 Effects of PUGNAc on PKC translocation

The results obtained from PUGNAc treatment of SVG cells were similar to those obtained after STZ treatment. As with STZ treatment, PUGNAc treatment reduced PKC-α and PKC-ε in the membrane fractions and at a similar rate while not significantly changing PKC-βII and PKC-ι membrane concentrations (Figure 4.5). PKC-α was reduced by 66%±8% (p<0.005) nine hours after the addition of PUGNAc (Figure 4.5). With PKC-ε, a 40%±12% (p<0.02) reduction was detected five hours after treatment and a 73%±7% (p<0.0005) reduction after nine hours (Figure 4.5).

4.2.5 Effects of NAGBT on PKC translocation

Unlike treatment with STZ or PUGNAc, NAGBT treatment resulted in no reduction in membrane associated PKC-α throughout the treatment period and only a modest (13%±1.5%) after 9 hours of treatment (Figure 4.6). PKC-βII and PKC-ι membrane concentration did not deviate from control levels throughout the time course and cytosolic levels of all isoforms analyzed also did not change from control levels following NAGBT treatment (Figure 4.6).

4.3 Discussion

Previous work in adipocytes and kidney cells has demonstrated that increased flux through the HBP can alter the overall activity and translocation of certain PKC isoforms [3-7,22]. While the specific changes vary among the experimental systems it has been hypothesized that these alterations in PKC may be the result of increases in the O-
SVG cells were treated with 80µM PUGNAc for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Membrane fractions were treated with isoform specific anti PKC antibodies. Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for membrane fractions probed with anti PKC-βII, PKC-ι, PKC-α, and PKC-ε antibodies are shown (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01

Figure 4.5 - Effects of PUGNAc on PKC isoforms in membrane fractions.
SVG cells were treated with 70µM NAGBT for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Membrane fractions were treated with isoform specific anti PKC antibodies. Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for membrane fractions probed with anti PKC-βII, PKC-ι, PKC-α, and PKC-ε antibodies are shown (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01.
GlcNAc modification on certain intracellular proteins [5,22]. To investigate this hypothesis, we examined the effects of elevated O-GlcNAc levels on the translocation of specific PKC isoforms in an SV40-transformed human astroglial cell line. Increases in cellular O-GlcNAc were achieved by increasing flux through the HBP with glucosamine [23] or inhibiting O-GlcNAcase with STZ, PUGNAc, and NAGBT [16,17,19,20,24,25]. A comparison of all four compounds demonstrates their usefulness as tools for modulating O-GlcNAc levels while also revealing their quantitative and kinetic differences in O-GlcNAc protein modification. These differences can, as discussed later, result in variations in protein regulation and possibly other intracellular functions related to this modification.

While a link between O-GlcNAc and PKC isoform regulation has not been investigated the effects of glucosamine on these enzymes has been well documented. Studies demonstrating a connection between increased glucosamine and PKC regulation have, however, produced a wide variety of results. Therefore, after confirming that glucosamine increases O-GlcNAc in our system, we investigated the effect of this treatment on the translocation of four PKC isoforms to the cell membrane of SVG cells. We examined the translocation of two conventional isoforms (PKC-α and PKC-βII), one novel isoform (PKC-ε), and one atypical isoform (PKC-ι). In our 8 mM glucosamine treated samples, a relatively rapid increase in membrane associated PKC-βII was observed (41%±13% one hour and 73%±8% three hours post treatment). A similar translocation of PKC-β was observed by Kolm-Litty et al. [6] in kidney mesangial cells following treatment with 12 mM glucosamine. In addition, we observed a relatively slow decrease of PKC-ε from the membrane (48%±15% decrease nine hours post treatment)
while PKC-α and PKC-ι levels remained unchanged throughout the treatment period. Interestingly, these results are in contrast to Kolm-Litty’s findings that demonstrate an increased translocation of both PKC-α and PKC-ε in response to glucosamine treatment [6]. In addition, other groups examining PKC observed both similar and contrasting results following glucosamine treatment. Experiments by Goldberg et al. [5] also in kidney mesangial cells but with a much lower (2mM) glucosamine concentration did not find any changes in PKC isoform translocation; however, they did observe an increase in PKC-βI, PKC-δ, and PKC-ε enzyme activity. Furthermore, adipocytes treated with 3mM glucosamine [3] showed an increased translocation of PKC-ε but no changes in PKC-α or PKC-β. While complex, it is widely recognized that the same PKC isoforms often have different or even opposing roles in different cell types [26]. These cell type differences most likely explain some of these variations in results while the varying concentrations of glucosamine used in these experiments likely contribute to other observed differences. All of this data, however, emphasizes a connection between increased glucosamine levels and PKC regulation and indicate that this connection occurs in a wide variety of cell types but with unique characteristics.

To investigate if results obtained following glucosamine treatment were specifically due to elevated O-GlcNAc, intracellular O-GlcNAc levels were increased by an alternative mechanism using the O-GlcNAcase inhibitors STZ, PUGNAc, and NAGBT. To our knowledge this study is the first to examine the effects of all of these inhibitors on PKC isoform translocation. Similar to glucosamine treatment, cellular treatment with STZ and PUGNaC resulted in both increases in global O-GlcNAc levels and large, significant decreases in membrane associated PKC-ε levels. The results
obtained following treatment with NAGBT, however, were not consistent with this trend. Whereas NAGBT treatment produced large increases in total O-GlcNAc levels similar to those observed following PUGNAc treatment, it yielded a relatively small but reproducible decrease in membrane bound PKC-ε (13%±1.5%). The reason for the divergent results obtained following NAGBT treatment is not known; however, there are two possibilities. The first possibility is that the recently characterized NAGBT may have other unknown cellular effects that may counteract or delay any O-GlcNAc induced decreases in membrane bound PKC-ε. This explanation is likely because to date NAGBT has only been used in one published paper [24] and, other than its ability to inhibit O-GlcNAcase and increase global O-GlcNAc levels in COS-7, its effects on other cellular pathways or systems have not been examined. A second, less likely, possibility for these results is that the large decreases in membrane bound PKC-ε observed following glucosamine, STZ, and PUGNAc treatments are the result of a shared pathway not related to the O-GlcNAc modification. This explanation seems unlikely because these compounds have been used extensively in a variety of systems and, other than increasing O-GlcNAc modification levels, are not known to affect another common pathway. Furthermore, although glucosamine and STZ have been demonstrated to produce oxidative stress \textit{in vitro} [27-29], PUGNAc treatment has been shown to have an opposite, protective effect against various stressors in several cell types [30]. PUGNAc has also been demonstrated not to mimic many of the cytotoxic effects of STZ in pancreatic cells [19]. Finally, unlike glucosamine and STZ, PUGNAc treatment does not alter phosphorylation of the serine/threonine kinase Akt providing further evidence against a shared pathway unrelated to O-GlcNAc (discussed further in chapter 8).
Whereas treatment with all four O-GlcNAc modulating agents resulted in significant although varying decreases in membrane associated PKC-ε, only STZ and PUGNAc treatments produced significant decreases in membrane bound PKC-α within the examined time period. Additionally, unlike membrane associated PKC-ε levels that showed significant decreases after 5 hours of STZ and PUGNAc treatment, significant decreases in PKC-α membrane levels were not observed until 9 hours post treatment. This evidence suggests that the membrane association of PKC-α is less sensitive to increases in O-GlcNAc protein modification and thus decreases more slowly than PKC-ε. It is also possible that the decreased levels of membrane bound PKC-α in response to STZ and PUGNAc treatments occur via a pathway not related to O-GlcNAc that is not affected by glucosamine or NAGBT. Further investigation, including treatment periods longer than 9 hours, is necessary to more conclusively determine a possible relationship between O-GlcNAc and PKC-α.

Unlike glucosamine, neither STZ, PUGNAc, nor NAGBT resulted in increased translocation of PKC-βII. This result suggest that the effect of glucosamine on PKC-βII may not be linked to the increases in O-GlcNAc but due to alternate glucosamine effects such as certain oxidative stressors [27], calcium entry [31], altered endoplasmic reticulum function [32], or other yet unidentified pathway and thus not affected by STZ, PUGNAc, or NAGBT (further discussed in Chapter 8). Also, none of the four treatment conditions had any significant effect on membrane associated levels of the atypical PKC-ι, further underscoring the complex and varying regulation of the PKC family.
The use of a variety of O-GlcNAc modulating agents in this study provides evidence that the O-GlcNAc modification may play a previously unknown role in regulating levels of PKC-ε and possibly PKC-α at the cell membrane. Since PKC translocates to the cell membrane upon activation [33], the observed decreases in membrane bound PKC-ε and PKC-α in response to increases in O-GlcNAc correspond to a decrease in their activity. Similarly, Akt, a serine-threonine kinase that, like PKC, depends on a series of ordered phosphorylations [34-38] and membrane translocation [34] for activation, undergoes a decrease in activity in response to increased O-GlcNAc levels in adipocytes [39]. Additionally, evidence supporting a selective coordination in the regulation of PKC-α and PKC-ε has been observed in other systems. In U251N glioma cells, PKC-α and PKC-ε are the only isoforms to translocate to the cell membrane in response to phorbol ester stimulation [40] and, in other cell types, these two isoforms have been shown to act cooperatively in the regulation of c-Jun N-terminal kinase (JNK) pathway [41] and in enhancing cell cycle progression [42].

The exact mechanism(s) by which O-GlcNAc may elicit changes in membrane associated PKC isoform levels remains to be determined. It is unlikely that these membrane associated decreases involve a disruption in the net synthesis of PKC-α or PKC-ε because no alterations in the larger cytosolic pool of these isoforms were observed. Alternatively, prolonged activation of PKC isoforms has been shown to result in their down-regulation from the membrane [40,43]; however, since increased translocation of PKC-α and PKC-ε was not observed prior to the observed decreases, it is unlikely that increases in activators such as DAG or phospholipids account for our data. Since there is no evidence to suggest that PKC is directly modified by O-GlcNAc [5], the
effects on these PKC isoforms may be mediated by O-GlcNAc modification of proteins that regulate their activation and/or degradation.

There are several points at which increases in O-GlcNAc modifications could elicit the observed decreases in membrane associated PKC-α and PKC-ε. First disrupting interactions with isoform-selective PKC binding proteins, such as receptors for activated C kinase (RACKs) could reduce membrane association. For instance, disrupting the association between RACK1 and PKC-βII has been shown to block its translocation to the plasma membrane [44] and a RACK specific to PKC-ε (RACK2) has been identified [45] and shown to play a critical role in this isoform’s translocation [46]. Korzick et al. [47] showed that an age-related decrease in the translocation of PKC-α and -ε in rat myocardial cells following α-adrenergic receptor stimulation was related to a decrease in RACK1 and RACK2 levels [47]. Second, altering activation of upstream enzymes, such as phosphoinositide 3’-OH-kinase (PI3-K), known to be involved in the PKC activation cascade could prevent translocation. While short (2 hour) glucosamine treatment has been shown to increase PI3-K activity in adipocytes [3], longer (> 4 hour) glucosamine treatment in skeletal muscle [48] and direct PI3-K modification with O-GlcNAc in endothelial cells [1] correlates with reduced activity. Since PI3-K activates 3’-phosphoinositide-dependent kinase-1 (PDK-1) that can in turn phosphorylate and activate conventional [49], novel [50,51], and atypical [52] PKC isoforms, an elevation of O-GlcNAc affecting PI3-K could reduce PKC activation. Third, increased O-GlcNAc could block the “recycling” of PKC-α and PKC-ε back into the active PKC pool. Upon dephosphorylation PKC is deactivated and removed from the plasma membrane and is either degraded by proteolysis or stabilized by binding to Hsp70 [53]. Once stabilized,
PKC can be rephosphorylated, reactivated, and returned to the plasma membrane. Hsp70 has been shown to be modified by O-GlcNAc [54] and, therefore, increased O-GlcNAc may alter its ability to bind and prevent proteolysis of select PKC isoforms.

In summary, our data in SVG cells demonstrates a novel link between increased levels of O-GlcNAc and decreases in active, membrane associated PKC-ε and possibly PKC-α. Furthermore, glucosamine treatment resulted in the activation of PKC-βII via a pathway likely unrelated to the O-GlcNAc modification while PKC-τ was unaffected by this modification.
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Chapter 5

Investigation of potential mechanisms for decreases in membrane associated PKC-ε and PKC-α

5.0 Introduction

Treatment of SVG cells with the O-GlcNAc modulating agents STZ and PUGNAc resulted in dramatic decreases in the levels of membrane associated PKC-ε and -α, while not affecting membrane associated PKC-βII or -ι (see chapter 4). These results suggest that this isoform-specific reduction is related to the O-GlcNAc modification of certain proteins associated with the regulation of these PKC isoforms. As discussed in Chapter 4, there are several possible points at which O-GlcNAc protein modification may affect the levels of these isoforms at the cell membrane. Three of these possible interaction points are the inhibition of upstream PKC-ε and PKC-α activators, RACK mediated PKC isoform translocation, and the Hsp70 mediated recycling of these isoforms. First, PI 3-K, an upstream indirect activator of PKC isoforms, has been shown to be directly modified by O-GlcNAc and this modification correlates with a reduction in its activity [1]. Therefore, a decrease in the activity of a PKC activator such as PI 3-K could result in decreased PKC isoform activation manifesting in a decrease in the translocation of these isoforms to the plasma membrane. Second, although a relationship between RACKs and the O-GlcNAc modification has not been investigated, the association between the PKC-βII and RACK1 and PKC-ε and RACK2 has been
demonstrated to facilitate PKC translocation to the plasma membrane [2-4]. Since the disruption of this interaction results in the decreased translocation of the PKC isoform [3], and the O-GlcNAc modification is known to affect protein-protein interactions (section 1.4.3), it is possible that a misregulation of this step is responsible for the decreases in membrane associated PKC-α and -ε observed in our system. Third, Hsp-70, an O-GlcNAc modified protein [5], has been demonstrated to bind and stabilize dephosphorylated PKC-βII and -ζ [6]. This binding allows the isoforms to be rephosphorylated and recycled back to the plasma membrane [6]. Therefore, the sustained O-GlcNAc modification of Hsp-70 may disrupt its interactions with PKC-α and PKC-ε resulting in decreased recycling and manifesting in decreased membrane associated levels and increased degradation of these isoforms. The focus of this chapter is to examine if increased levels of intracellular O-GlcNAc modification affects either the activation and/or translocation of PKC-ε and -α or their Hsp70 mediated recycling.

5.1 Increased O-GlcNAc and PKC-α and -ε translocation

In order to investigate if increased O-GlcNAc protein modification decreased membrane-associated levels of PKC-α and -ε by either disrupting the phosphorylation of these isoforms or their interactions with RACKs, SVG cells were pretreated for 5 hours with 5mM STZ and then treated with phorbol 12-myristate-13-acetate (PMA). Cells were treated for 5 hours with 5mM STZ because this treatment condition was demonstrated significantly increase global O-GlcNAc levels and also be sufficient to facilitate a 48% decrease in membrane associated PKC-ε. Longer (9 hour) preincubation of cells with STZ prior to stimulation with PMA (up to 5 hour treatment) was avoided in
order to reduce any toxic effects brought about by prolonged STZ exposure [7,8]. PMA was chosen to induce PKC translocation because it is a well characterized activator of both cPKC and nPKC isoforms. In order for PMA to induce translocation, PKC must first be fully phosphorylated [9] (Section 2.3.1). Additionally, blocking the association between PKC-βII and RACK1 [3] or PKC-ε and RACK1 [10] has been shown to prevent the PMA-induced translocation of these isoforms to the plasma membrane or membrane associated focal adhesions respectively. Therefore, if either the activity of an upstream PKC activator or its ability to associate with a RACK is disrupted after pretreatment with 5mM STZ for 5 hours, a reduction in PMA-induced translocation of the isoforms should be observed. In order to determine the normal translocation pattern of PKC isoforms in this cell type to PMA, cells were also treated with 100nM PMA without STZ pretreatment.

5.2 Increases O-GlcNAc and Hsp70 mediated PKC recycling

In addition to a disruption in PKC isoform phosphorylation or RACK association, reduction in membrane PKC-α and -ε levels could be caused by the disruption of Hsp70-mediated PKC recycling. In order to investigate this hypothesis, immunoprecipitation was used to first analyze if either PKC-α or -ε associated with Hsp70 in SVG cells and, if so, if this interaction was disrupted under conditions of increased O-GlcNAc protein modification. Additionally, since a disruption in Hsp70 mediated PKC recycling correlates with increased PKC proteolysis and partitioning to the cytoskeletal fraction [6] (see section 2.4), cytoskeletal fractions from cells treated with or without O-GlcNAc modulating agents were analyzed for increased PKC isoform degradation.

5.3 Materials and Methods
Most materials and methods used in this chapter can be found in section 4.1. Materials and methods used solely in this chapter are described below.

5.3.1 Cell culturing and sample preparation for PMA treatment

SVG cells were cultured as described previously. In order to determine the PKC isoform translocation pattern in response to PMA, SVG cells were treated with 100nM PMA in DMSO or in an equal volume of DMSO alone for 15 minutes, 1 hour, 5 hours, or 21 hours. To analyze any effects of increased O-GlcNAc on PMA stimulated PKC translocation, cells were treated with or without 5mM STZ for 5 hours. After the 5 hour preincubation period, cells were treated with 100nM PMA in DMSO or with an equal volume of DMSO alone for 15 minutes, 1 hour, or 5 hours without changing the medium. Longer time points (> 10 hours total STZ incubation) were not done because long incubations with STZ have been shown to induce DNA damage in pancreatic β-cells [7,8]. After the appropriate treatments, cells were fractionated into cytosol and membrane fractions and analyzed by SDS-PAGE and western blotting using isoform specific PKC antibodies as described in section 4.1.4.

5.3.2 Cell culturing and sample preparation for immunoprecipitation

SVG cells were cultured as previously described. Samples were treated with or without 5mM STZ for 9 hours where appropriate. When cells reached approximately 90% confluency, cells were washed two times in cold PBS and scraped into 3ml of cold IP buffer (20mM Tris base (pH 7.4), 1mM EDTA, 1mM EGTA, 2mM MgCl₂, 150mM NaCl, 1mM DTT, 1mM PMSF, 1mM Na₃VO₄, 4µg/ml aprotinin, 1.0% Triton X-100). Samples were incubated on a rocker at 4°C for 10 minutes followed by sonication two times for 3 seconds at 7 watts. All steps were performed at 4°C unless otherwise stated.
Samples were centrifuged at 12000xg (12500rpm) for 10 minutes and the supernatant was removed. The pellet was resuspended in 1x SDS-PAGE sample buffer, boiled for 5 minutes and analyzed as the cytoskeleton fraction. Supernatant protein concentrations were determined by Bradford assay using BSA as a standard (section 4.1.3). Samples were then diluted to equal protein concentrations (1.5-1.0mg/ml) by adding an appropriate amount of IP buffer. Samples were precleared with immobilized Protein A agarose (Pierce Biotechnology, Rockford, IL, catalog # 22811) by adding 25µl of beads to approximately 1.0ml of sample in a 1.5ml centrifuge tube. Samples were then incubated for 1 hour with gentle rocking. Samples were then centrifuged at 1000xg for 5 minutes and supernatant was removed. The pellet, containing proteins nonspecifically bound to the Protein A agarose beads, was resuspended in 1x SDS-PAGE sample buffer, boiled for 5 minutes, and supernatant was removed for later analysis.

Following preclearing, appropriate primary or control antibody were added to each sample. Primary antibodies used for immunoprecipitation include anti-nPKC-ε (Santa Cruz Biotechnology, sc-214), anti-cPKC-α (Santa Cruz Biotechnology, sc-8393), and anti-Hsp70 (Stressgen, SPA-810) whereas antibodies used as controls include normal rabbit IgG (Santa Cruz Biotechnology, sc-2027), normal mouse IgG1 (Santa Cruz Biotechnology, sc-3877), anti-p27 (Santa Cruz Biotechnology, sc-1641), and anti-Mat1 (Santa Cruz Biotechnology, sc-13142). A variety of antibody amounts (5.0µg - 0.5µg) were used for immunoprecipitation experiments with 1.0µg found to give the best signal to noise ratio following western blot analysis. Samples with antibodies were incubated overnight with gentle rocking. The next morning, 10µl of immobilized Protein A agarose was added per 1µg of antibody and samples were incubated for 2 hours with gentle
rocking. Samples were then centrifuged at 1000xg for 5 minutes, supernatants were removed and retained at -70°C, and beads were washed 4 times in IP buffer. Beads were then resuspended in 25µl of 3x SDS-PAGE sample buffer, boiled for 5 minutes, and supernatant was removed. In order to remove the small volume (~25µl) of sample from beads, a small hole was punctured in the bottom of the centrifuge tube with 6 beading 1¼ inch sewing needle. The punctured tube was then placed inside of another tube and briefly centrifuged. The liquid passed through the hole into the new tube leaving the beads in the punctured tube. Samples were then analyzed by SDS-PAGE, as described in section 4.1.4, using specified antibodies.

After initial immunoblot was complete, the nitrocellulose membrane was subjected to a second immunoblot procedure. The antibodies used for this immunodetection step were the same as the antibody used for immunoprecipitation. This process was done to ensure that the immunoprecipitation had effectively precipitated the desired protein. First, the nitrocellulose membranes were washed 2 times for 15 minutes in TBST and then placed in stripping solution (200mM glycine pH 2.2) for 45 minutes. Membranes were then washed 1 time for 15 minutes in TBST, blocked in 5% nonfat dry milk in TBST, immunoblotted as described in section 4.1.4.

5.4 Results

5.4.1 Effects of increased O-GlcNAc on PKC isoform translocation

As expected, cellular treatment with 100nM PMA in DMSO had a dramatic effect of the translocation of PKC-α, -ε, and -βII but did not affect either cytosolic or membrane levels PKC-ι when compared to DMSO treatment alone (Figure 5.1). Because PMA activates PKC by binding to the C1 domain (section 2.1), isoforms lacking a
Effects of PMA Treatment on PKC Isoforms

Figure 5.1 – Effects of 100nM PMA treatment on PKC isoform translocation. SVG cells treated with 100nM PMA in DMSO for 15min (T=0.25), 1 hour (T=1), 5 hours (T=5), or 21 hours (T=21). Additionally, cells were treated with an equivalent volume of DMSO alone for 1, 5, or 21 hours (control). Samples were then fractionated into membrane and cytosol fractions.
functional C1 domain, such as the aPKC’s, are not affected by PMA treatment thus providing an explanation for the unresponsiveness of PKC-ι to the treatment. Unlike PKC-ι, after only 15 minutes of treatment, all detectable PKC-ε had translocated from the cytosol to the membrane fraction. Also after 15 minutes of treatment, most PKC-βII and about half of PKC-α had translocated. After 5 hours of PMA treatment, membrane associated levels of conventional PKC-α and -βII began to decrease and were not detectable after 21 hours of treatment. Membrane associated levels of the novel PKC-ε did not begin to decline until 21 hours of treatment. These results are consistent with PMA-induced PKC isoform translocation pattern observed by Besson et al. [10] in glioma cells.

In SVG cells pretreated with 5mM STZ for 5 hours followed by treatment with 100nM PMA for 15min, 1 hour, or 5 hours, no significant alterations in the PKC isoform translocation was observed (Figure 5.2). Twenty-one hours of PMA treatment was not done because this would require a total of 26 hours of incubation with STZ. Such long exposures of cells to STZ has been shown to be toxic in pancreatic β-cells [7,8].

5.4.2 Effects of increased O-GlcNAc on PKC recycling

In order to investigate the effects of increased O-GlcNAc protein modification of PKC recycling, SVG cells were first treated with or without 5mM STZ for 9 hours. These treatment conditions had been shown to decrease the level of membrane associated PKC-α and -ε with a more dramatic effect on PKC-ε. To determine if this treatment condition increased the degradation of PKC-ε, a sign that PKC recycling is disrupted [6], cytoskeleton fractions prepared as described in section 5.3.2 were analyzed by SDS-
Figure 5.2 – Effects of 5mM STZ for 5 hours treatment on PMA induced PKC isoform translocation. SVG cells were pretreated with 5mM STZ for 5 hours followed by stimulation with 100mM PMA in DMSO for 15 min (T=0.25), 1 hour (T=1), or 5 hours (T=5). Additionally, cells were pretreated with 5mM STZ for 5 hours followed by addition of an equivalent volume of DMSO for 15 min (control 15min), 1 hour (control 1hr), or 5 hours (control 5hr). An untreated sample was also prepared (untreated). Samples were then fractionated into cytosol and membrane fractions. Only membrane fractions are shown.
PAGE and western blotting using an anti-PKC-ε polyclonal antibody (Santa Cruz Biotechnology, sc-214). Results showed increased levels of 45kDa and 30kDa degradation fragments in samples treated with STZ compared with untreated samples (Figure 5.3).

Because Hsp70 has been shown to bind to PKC isoforms following their dephosphorylation thus preventing their degradation [6], the observed increase in PKC-ε degradation following STZ treatment suggests a possible disruption in its recycling in response to increased O-GlcNAc protein modification. To investigate if either PKC-α or PKC-ε interacts with Hsp70 in SVG cells and, if so, if this interaction was altered by increases in total cellular O-GlcNAc, association between these isoforms and Hsp70 was investigated by immunoprecipitation. Neither immunoprecipitation of PKC-α (Figure 5.7) nor PKC-ε (Figures 5.4 and 5.5) showed an association with Hsp70 or immunoprecipitation of Hsp70 showed an association with the two PKC isoforms under experimental conditions in our cell system (Figures 5.4, 5.5, and 5.7). Additionally, no interaction between PKC-ε and Hsp70 was observed after increasing O-GlcNAc levels by cellular treatment with 5mM STZ for 9 hour (Figure 5.6). For immunoprecipitation experiments performed using anti-PKC-α (mouse monoclonal IgG1), negative control immunoprecipitations were done using a nonspecific mouse monoclonal IgG1 and, because p27 and Hsp70 have been shown to interact in rat thyroid cells [11], anti-p27 was used as a positive control antibody for immunoprecipitations experiments (see section 5.3.2) (Figure 5.7). Additionally, an immunoprecipitation was done with an anti-ménage a trois 1 protein (Mat1) mouse monoclonal IgG1 antibody. Interestingly, a relatively
Figure 5.3 – Effects of 5mM STZ on PKC-ε cleavage. SVG cells were treated with 5mM STZ for 9 hours (+STZ) or untreated (-STZ). Cells were then fractionated into cytoskeletal fractions (cytoskeleton) and soluble fractions (supernatant).
PKC-ε and Hsp70 Association

**Figure 5.4 – PKC-ε and Hsp70 Association.** SVG cells were lysed and immunoprecipitated using anti-PKC-ε antibody (IP PKC-ε) or normal rabbit IgG (IP control IgG). Samples were then western blotted for Hsp70. The same membrane was then stripped and probed for PKC-ε to ensure immunoprecipitation was effective.
Hsp70 and PKC-ɛ Association

**Figure 5.5 – Hsp70 and PKC-ɛ Association.** SVG cells were lysed and immunoprecipitated using anti-Hsp70 antibody (IP Hsp70) or normal mouse IgG (IP control IgG). Samples were then western blotted for PKC-ɛ. The same membrane was then stripped and probed for Hsp70 to ensure immunoprecipitation was effective.
Increased O-GlcNAc and PKC-ε/Hsp70 Association

**IP: PKC-ε**

**Western Blot: Hsp70**

**Figure 5.6 – Increased O-GlcNAc and PKC-ε/Hsp70 Association.** SVG cells treated with 5mM STZ for 9 hours (+STZ) or untreated (-STZ) followed by immunoprecipitation using anti-PKC-ε antibody (IP PKCε) or normal rabbit IgG (IP IgG). Supernatants from immunoprecipitations treated with STZ and IP’d for PKC-ε (supernatant 1) or normal rabbit IgG (supernatant 2), or untreated and IP’d for PKC-ε (supernatant 3) or normal rabbit IgG (supernatant 4) were also analyzed. Samples were then western blotted for Hsp70.
PKC-α and Hsp70 Association

**Figure 5.7 – PKC-α and Hsp70 Association.** SVG cells were lysed and immunoprecipitated using anti PKC-α antibodies (IP PKC-α), mouse IgG1 (IP mouse IgG1), anti-p27 (IP p27), Mat1 (IP Mat1), or Protein A beads with no antibody (beads only). Samples were then western blotted for Hsp70.
strong interaction between Mat1 and Hsp70 was observed that has not been previously
documented in the literature (Figure 5.7).

5.5 Discussion

Disruptions in PKC phosphorylation [9] or a disruption in the interaction between
either PKC\(\beta\)II [3] or PKC-\(\varepsilon\) [10] and RACK1 have all been shown to decrease the
translocation of PKC isoforms in response to PMA stimulation. Therefore, as indicated
by the results, the decreases in membrane associated PKC-\(\alpha\) and PKC-\(\varepsilon\) under conditions
of increased O-GlcNAc did not appear to be due to either a disruption in the isoforms’
ability to translocate due to altered isoform phosphorylation or its ability to bind RACK.
Results showing increased degradation of PKC-\(\varepsilon\) following pretreatment with STZ
indicated that the previously observed decreases in membrane associated PKC-\(\varepsilon\) and -\(\alpha\)
may be due to an increased rate of proteolysis. The lack of detectable association
between either PKC-\(\alpha\) or PKC-\(\varepsilon\) and Hsp70 suggest that this increased proteolysis in not
a result of the Hsp70-mediated PKC recycling pathway.

One alternate pathway that could be responsible for the increase in PKC-\(\varepsilon\)
degradation under conditions of increased O-GlcNAc modification is the increased action
of specific phosphatases. It has been shown that dephosphorylation of conventional PKC
isoforms precedes their degradations [12] and down regulation of PKC-\(\alpha\) by
dephosphorylation can be a protein phosphatase 1 (PP1) and 2A (PP2A) mediated event
[13]. Additionally, OGT, the enzyme that adds O-GlcNAc to proteins, exists in stable
and active complexes with the serine/threonine phosphatases PP1-\(\beta\) and PP1-\(\gamma\) [14]
suggesting that phosphatase activity may be linked to the regulation of the O-GlcNAc
modification. Therefore, it is possible that increases in global O-GlcNAc levels result in increased phosphatase activity resulting in increased PKC isoform dephosphorylation and degradation. The isoform specificity may be due to specific co-compartmentalization of PKC-α and -ε with the specific protein phosphatase [15] as both PP2A [16] and PP1-β and -γ [17] have been shown to localize to specific and distinct cytoskeletal locations within neurons. Also, the decreases in membrane bound, and not cytosolic, PKC-α and -ε could be explained by the fact that membrane bound PKC is two orders of magnitude more sensitive to dephosphorylation than the soluble form [18]. Investigations, using specific phosphatase inhibitors in conjunction with STZ and PUGNAc, could help determine if this mechanism is responsible for the observed decreases in membrane associated PKC-α and -ε.
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Chapter 6

PKC-α and PKC-ε downregulation and apoptosis induction

6.0 Introduction

Research discussed previously in chapter 4 demonstrated that increased levels of the O-GlcNAc protein modification resulted in decreases in membrane associated PKC-ε and possibly PKC-α. Research by others has indicated a link between these two PKC isoforms and promotion of apoptosis [1-3]. This chapter investigates whether decreases in the membrane associated, active forms of PKC-α and -ε result in increased apoptosis in SVG cells.

6.1 Apoptosis

Apoptosis is a programmed form of cell death with typical cell morphology including membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation [4]. Also, the activation of cysteine-dependent aspartate-directed proteases called caspases responsible for much of the apoptotic related proteolytic cleavage is another hallmark of apoptosis [5]. Unlike cell death by necrosis that typically occurs due to severe hypoxia, extreme temperatures, or mechanical trauma, apoptosis is a tightly regulated, energy requiring process that has been highly conserved throughout evolution [4]. Currently, there are two well studied apoptotic pathways responsible for the activation of the caspases. One pathway is initiated by the binding of a ligand to its transmembrane death receptor that in turn recruits and activates certain caspases [6]. The
other pathway involves the release of caspase-activating proteins from the mitochondria into the cytosol forming an apoptosome that can bind and activate select caspases [7].

The caspases are proteins synthesized in normal cells as proenzymes [8]. Following the appropriate signal, these proenzymes can be rapidly activated by autoproteolytic cleavage or cleavage by another caspase at specific aspartic acid residues [8]. There are currently 14 known members of the caspase family of which 7 mediate apoptosis [8]. In general, caspases with long pro-domains function as upstream, initiator, caspases and are capable of proteolytically activating downstream, effector, caspases which contain shorter pro-domains [8]. The effector caspases act on a variety of substrates resulting in proteolysis of cellular proteins ultimately resulting in apoptotic cell death. All caspases specifically recognize and cleave a tetrapeptide sequence on their substrate with an absolute requirement for an Asp residue. The best characterized caspase substrate is poly-(ADP-ribose) polymerase (PARP), a nuclear protein implicated in DNA repair, which is cleaved into characteristic 89kDa and 24kDa fragments [8]. PARP is one of the earliest proteins targeted for specific cleavage and is commonly used as a marker for the initiation of apoptosis [8]. Other caspase substrates include ICAD (inhibitor of caspase-activated DNAse) that is cleaved and activated allowing CAD to translocate to the nucleus where it is responsible for internucleosomal DNA cleavage [9,10]. Also, caspase cleavage of lamins results in nuclear shrinkage whereas cleavage of cytoskeletal proteins like fodrin and actin leads to cytosolic reorganization [11-13]. Furthermore, caspase-dependent cleavage of DNA-protein kinase, cell cycle regulators, transcription factors, and cell signaling proteins have also been reported [14-18]. The
caspases are also responsible for cleaving pro-apoptotic proteins Bid and Bax and cell survival factors Bcl-2 and Bcl-XI during apoptosis [19-21].

One well studied method for caspase activation is through ligand binding to plasma membrane receptors belonging to the tumor necrosis factor (TNF) receptor superfamily. This family includes Fas, TNF receptor-1, death receptor-3, TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R1), TRAIL-R2, and DR6. The well studied Fas receptor is activated by the binding of the Fas ligand (FasL) that induces trimerization and recruitment of specific adaptor proteins [22,23]. The Fas receptor contains a death domain (DD) in its cytoplasmic region that interacts with the adaptor protein Fas-associated death domain protein (FADD), forming a death receptor-induced signaling complex [24,25]. FADD also contains a death effector domain (DED) that binds procaspase-8 (Medema, 1997). Once bound, procaspase-8 is proteolytically activated to active caspase-8, which in turn can activate downstream effector caspases [8].

A second caspase-activating apoptotic pathway involves the participation of the mitochondria. The process involves the formation of the “apoptosome” in which cytochrome C released from the mitochondria interacts with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 in the presence of ATP [26]. The release of these proteins from the mitochondria is a result of dramatic mitochondrial membrane depolarization. This depolarization occurs following the formation of the mitochondrial permeability transition pore (PTP) that is composed of adenine translocator at the inner membrane and voltage-dependent anion channel (VDAC) at the outer membrane. The formation of this complex results in the activation of caspase-9 that in turn activates
caspases-3, 6, and 7 [27]. One group of proteins that influence the opening of the PTP is the Bcl-2 family of proteins. The Bcl-2 family of proteins includes pro-apoptotic members, such as Bax, Bad, Bak, and Bim, and anti-apoptotic members such as Bcl-2 and Bcl-X\textsubscript{L}. Pro- and anti-apoptotic Bcl-2 proteins are able to heterodimerize and suppress each other’s function; however, a single mechanism by which these proteins regulate apoptosis has not been completely determined. Both pro- and anti-apoptotic Bcl-2 proteins are subject to posttranslational modifications, such as phosphorylation, which affects their death or survival promoting functions [4].

6.2 Protein Kinase C and apoptosis

Early experiments investigating the potential role of PKC isoforms in apoptosis yielded contradictory results indicating PKC to be both pro-and anti-apoptotic [28,29]. With the development of isoform-specific activators and inhibitors it became possible to investigate the role of individual isoform’s roles in apoptotic regulation. Two PKC isoforms thus far implicated in cell survival are PKC-\(\alpha\) and PKC-\(\varepsilon\). Cellular depletion of PKC-\(\alpha\) using antisense oligonucleotides or phorbol ester-mediated downregulation has been shown to induce apoptosis in endothelial cells [30] and glioma cells [31,32]. Inactivation of PKC-\(\alpha\) has also increases apoptosis in liver cells [3]. Although the mechanism by which PKC-\(\alpha\) prevents apoptosis is not fully understood, two potential mechanisms include its phosphorylation of Bcl-2 and/or phosphorylation of Raf-1. Phosphorylation of the anti-apoptotic Bcl-2 protein stabilizes it and enhances its ability to prevent apoptosis [33,34]. Additionally, PKC-\(\alpha\) mediated phosphorylation of Raf-1 results in its localization to the mitochondrial membrane through an interaction with Bcl-2 [35] where it can then phosphorylate and inactivate the pro-apoptotic protein BAD [36].
Similar to PKC-α, PKC-ε is also widely accepted as having anti-apoptotic properties. Overexpression of PKC-ε in glioma cells inhibits TRAIL (tumor necrosis factor-α related apoptosis-inducing ligand) induced apoptosis [1]. PKC-ε inhibits apoptosis in prostate cancer cells by interacting with Bax [2]. Although the mechanism(s) responsible for its anti-apoptotic function is unknown, PKC-ε, like PKC-α, has been shown to activate Raf-1 [37].

Conversely, unlike PKC-α and -ε, PKC-δ is known to have pro-apoptotic functions. Many proapoptotic stimuli, such as signals initiated by the death receptor [38], ultraviolet radiation [39], and etoposide [40], have been demonstrated to result in the activation of PKC-δ. Additionally, it has been demonstrated that a loss of PKC-δ is associated with tumor growth [41]. The down-regulation of PKC-δ or overexpression of a kinase dead form of this enzyme provides a survival signal that prevents the induction of apoptosis in response to serum deprivation [42].

6.3 Materials and methods

6.3.1 Cell culture

SVG cells were cultured and treated with either 8mM glucosamine, 5mM STZ, 80µM PUGNAc, or 70µM NAGBT for 9 hours as described in section 4.1.2.

6.3.2 Cell fractionation

For glucosamine, STZ, and PUGNAc treated samples, whole cell homogenates were generated by first washing cells 2 times in ice cold PBS. Cells were then scraped into 900µl of homogenization buffer with 1.0% Triton X-100 (section 4.1.3) and transferred to 1.5ml centrifuge tubes. Samples were then sonicated 2 times for 5 seconds.
at 7 watts using a Fisher Scientific Sonic Dismembrator F60. Unlysed cells and large cellular debris was pelleted out by centrifuging at 1000xg for 5 minutes at 4°C. Supernatants were removed and labeled whole cell homogenates.

Due to a limited supply of NAGBT, whole cell homogenates treated with this inhibitor were not generated. Instead samples that had been previously fractionated into cytosol and membrane fractions as described in section 4.1.3 were analyzed for PARP cleavage.

6.3.3 Electrophoresis and Western blotting

Samples were analyzed for PARP/PARP cleavage fragment or procaspase-7/caspase-7 as described in section 4.1.4 with a few modifications. Anti-PARP mouse monoclonal antibody (catalog # sc-8007) and anti-caspase-7 goat polyclonal (catalog # sc-8510) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were both used at a 1:400 dilution in TBST with 3% nonfat dry milk. Goat anti mouse antibody conjugated with horseradish peroxidase (BioRad) or bovine anti-goat antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology catalog # sc-2350) were used in conjunction with the respective primary antibody at 1:10,000 dilutions also in TBST with 3% nonfat dry milk.

6.4 Results

6.4.1 Effects of PUGNAc and STZ treatment on PARP cleavage

As discussed in Chapter 4, treatment of SVG cells for 9 hours with either PUGNAc or STZ resulted in a large (~80%) decreases in membrane associated PKC-α and -ε whereas glucosamine and NAGBT treatments reduced PKC-ε to a much less extent and produced no significant decrease in PKC-α over the same time frame. To
determine if the large decrease in the anti-apoptotic PKC-α and -ε increased PARP cleavage, an early indicator of apoptosis, samples were treated with or without 5mM STZ or 80µM PUGNAc for 9 hours and then analyzed by SDS-PAGE and immunoblotting with anti-PARP antibody. This antibody has been demonstrated to recognize both the 112kDa whole protein and 85kDa cleavage fragment [43]. Cellular treatment with either STZ or PUGNAc for 9 hours resulted in an observable increases in the 85kDa PARP cleavage fragment with no observable change in uncleaved 112kDa PARP when compared to untreated control samples (Figure 6.1). As with previous experiments, there were no observable changes in cell morphology or total protein levels or loss of cell viability.

In order to determine if PARP cleavage was detectable at time frames earlier than 9 hours, SVG cells were treated with 80µM PUGNAc or 5mM STZ for 1, 3, 5, and 9 hours, prepared as described in 4.1.3, and analyzed by SDS-PAGE. When compared to untreated controls, increases in PARP cleavage were detectable after only 5 hours of treatment (Figure 6.2).

6.4.2 Effects of glucosamine and NAGBT treatment on PARP cleavage

As with PUGNAc and STZ treatment, SVG cells were treated with either 8mM glucosamine or 70µM NAGBT for 9 hours and analyzed for PARP cleavage by SDS-PAGE and western blot. Unlike PUGNAc and STZ treatment, no observable changes in PARP or PARP cleavage were observed following glucosamine or NAGBT treatment when compared to untreated control samples (Figure 6.1). Also, no changes in cell morphology or total protein levels or loss of cell viability were observed under these treatment conditions.
Increased O-GlcNAc protein modification and PARP cleavage

Western Blot:
PARP

Figure 6.1 – Increased O-GlcNAc protein modification and PARP cleavage
SVG cells were treated with or without 80µM PUGNAc (+PUGNAc and –PUGNAc respectively), with or without 5mM STZ (+STZ and –STZ respectively), with or without 8mM glucosamine (+GlcN and –GlcN respectively), or with or without 75µM NAGBT (+NAGBT and –NAGBT respectively). Cells were treated with each compound for 9 hours before preparing samples as whole cell homogenates (WCH) or membrane fractions. Whole cell homogenates of NAGBT were not prepared due to limited supply of inhibitor (see section 6.3.2). Samples were western blotted with anti-PARP antibody that recognizes both full length PARP and the 85kDa apoptotic cleavage fragment.
PUGNAc treatment and PARP cleavage

**Western Blot:**

**PARP**

<table>
<thead>
<tr>
<th>Control</th>
<th>T=1hr</th>
<th>T=3hr</th>
<th>T=5hr</th>
<th>T=9hr</th>
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**Figure 6.2 – PUGNAc treatment and PARP cleavage.** SVG cells were treated with 80µM PUGNAc for 1 hour (T=1hr), 3 hours (T=3hr), 5 hours (T=5hr), or 9 hours (T=9hr) or untreated (Control). Cells were lysed and western blotted with anti-PARP antibody that recognizes both full length PARP and the 85kDa apoptotic cleavage fragment.
6.4.2 Caspase-7 activation

Caspase-7 has been shown to cleave PARP in HL-60 and MCF-7 cells during apoptosis [44]. In order to determine if the PARP cleavage observed following STZ and PUGNAc treatments in SVG cells was associated with increased caspase-7 activation, cells were first treated with either 5mM STZ or 80µM PUGNAc or untreated for 9 hours. Samples were then analyzed by SDS-PAGE and western blotting with an anti-caspase-7 antibody that recognizes both the inactive, procaspase-7, form and the active, caspase-7, form of the enzyme [45]. Analysis revealed no detectable increase in active caspase-7 following STZ or PUGNAc treatment when compared to untreated controls (Figure 6.3)

6.5 Discussion

The results obtained indicate a strong correlation between decreases in membrane bound PKC-α and PKC-ε resulting from treatment with select O-GlcNAc increasing agents and increased PARP cleavage. Cellular treatment with PUGNAc and STZ produced large decreases in PKC-α and -ε and also corresponding increases in PARP cleavage whereas glucosamine and NAGBT produced smaller or no decreases in these isoforms and no observable increases in PARP cleavage. Examination of the time frames of PARP cleavage indicates that significant increases begin around 5 hours after treatment with STZ or PUGNAc. The fact that significant decreases in membrane bound PKC-ε but not PKC-α levels are detectable after 5 hours of STZ and PUGNAc treatment suggests that decreases in active PKC-ε may be primarily responsible for the PARP cleavage. Although glucosamine and NAGBT treatments do produce deceases in membrane associated PKC-ε, the level of decrease may not be large enough facilitate PARP cleavage. This data is the first to suggest a link between decreases in active PKC-
Increased O-GlcNAc and Caspase-7 activation

Western Blot:
Caspase-7
(reactive with pro-caspase-7 and p20 fragment

![Western Blot Image]

**Figure 6.3 – Increased O-GlcNAc and Caspase-7 activation.** SVG cells were treated with or without 5mM STZ (+STZ and −STZ respectively) or with or without 80μM PUGNAc (+PUGNAc and −PUGNAc respectively) for 9 hours. Cells were lysed and western blotted for Caspase-7 using an antibody that recognizes both the inactive Pro-caspase-7 and active Caspase-7 p20 cleavage product.
ε as a result of increases in the O-GlcNAc protein modification and induction of apoptosis.

Furthermore, Leverrier et al. [46] demonstrated that in rat pituitary adenoma cells apoptosis induced from cis-platinum and UV irradiation, but not after serum deprivation resulted in the limited proteolysis of PKC-α, -ε, and -δ. Following cis-platinum treatment and UV irradiation, DNA fragmentation appeared after 9 hours and significant PARP cleavage was observed after 16 hours. The proteolytic cleavage of the PKC isoforms resulted in the formation of a catalytic fragment of between 48 and 42kDa localizing in the particulate fraction. This cleavage was also shown to be calpain and caspase dependent [46]. As discussed in section 5.4.2, treatment of SVG cells for 9 hours with 5mM STZ resulted in increased formation of a 45kDa catalytic and 30kDa regulatory fragment in the cytoskeletal (detergent insoluble) fraction. Taken together with the results obtained from Leverrier et al. [46], it is possible that the increased cleavage of PKC-ε observed following increases in O-GlcNAc is a result of increased calpain and/or caspase activity. This process does not appear to involve the activation of caspase-7, however, and further investigation is needed to elucidate this pathway.
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Chapter 7

Akt

7.0 Introduction

Akt (also known as protein kinase B) is a family of serine-threonine kinases that play a major role in signal transduction activated by extracellular stimuli. There are three known Akt isoforms, Akt1, Akt2, and Akt3, each encoded by a separate gene. All three genes share greater than 85% homology. All three isoforms are assumed to have similar substrate specificity although conclusive testing has not been done and all three share similar regulation (discussed below). All three isoforms are ubiquitously expressed but the level of expression varies with tissue [1-3]. Akt1 is highly expressed in most tissues [4], Akt2 is largely expressed in insulin-responsive tissues [5], and Akt3 is most highly expressed in testis and brain [6,7].

7.1 Akt structural domains

All three Akt isoforms consist of an amino terminal pleckstrin homology (PH) domain, a central kinase domain, and a carboxyl-terminal regulatory domain containing a hydrophobic motif. The PH domain is comprised of approximately 100 amino acids and was originally found in pleckstrin, the major phosphorylation substrate for PKC in platelets [8]. The PH domain binds the lipid products phosphatidylinositol (3,4,5) trisphosphate (PIP3) produced PI3-K and phosphatidylinositol (3,4) bisphosphate (PIP2) and allows Akt to interact with cell membranes through these lipid products. Akt has
been shown to bind PIP3 and PIP2 with similar affinity [9,10]. The PH domain of Akt shares similarity with the PH domains of other proteins that bind 3-phosphoinositides [11,12].

The kinase domain of Akt is located in the central region of the protein and exhibits high homology to other AGC kinases such as PKC, protein kinase A (PKA), and p70S6K. All three isoforms have a 40 amino acid section at the carboxyl terminal end possessing an F-X-X-F/Y-S/T-Y/F (X is any amino acid) hydrophobic motif that is also share by most members of the AGC kinase family.

7.2 Akt activation

Like PKC, Akt is initially transcribed as an unphosphorylated single polypeptide chain. All three Akt isoforms undergo two phosphorylations to produce the stable, active enzyme. The translocation of Akt to the plasma membrane is a prerequisite for its phosphorylation [13]. Once at the membrane Akt1 is phosphorylated on Thr308 and Ser473, Akt2 is phosphorylated on Thr309 and Ser474, and Akt3 is phosphorylated on Thr305 and Ser472 [14] (Figure 7.1). The threonine residue is located in the activation loop and the serine residue is located in the hydrophobic motif. The phosphorylation of Akt on Thr308 (or equivalent residue) has been shown to be mediated by PDK1 [15,16] (Figure 7.2). The kinase responsible for the phosphorylation of Ser473 (or equivalent residue) is controversial and PDK1 [17], integrin-linked protein kinase [3], Akt [18], DNA-PK [19] and mTOR [20] are all potential candidates. It is generally accepted that, once at the plasma membrane, Akt is first phosphorylated on Ser473 [21-23]. The phosphorylation of Ser473 stabilizes the Akt and allows ATP binding [22]. The phosphorylation of the hydrophobic motif then promotes the phosphorylation of Thr308.
Akt Isoform Phosphorylation Sites

**Figure 7.1 – Akt Isoform Phosphorylation Sites**

PH- pleckstrin homology domain; catalytic – catalytic domain; regul. – C-terminus regulatory domain [14]
Akt Life Cycle

Figure 7.2 – Akt life cycle
PTEN – Phosphatase and Tensin homolog deleted on chromosome Ten,
PHLPP - PH domain Leucine-rich repeat Protein Phosphatase [24]
Following both phosphorylations, Akt detaches from the nucleus and translocates to the cytosol and nucleus [13].

The mechanism of Akt dephosphorylation and inactivation is largely unknown. Recently, a PH domain leucine-rich repeat protein phosphatase (PHLPP) was identified that specifically dephosphorylates the hydrophobic motif of Akt (Ser473 in Akt1) [24] (Figure 7.2). This PHLPP mediated dephosphorylation of Akt correlated with increased apoptosis and suppressing tumor growth in glioblastoma cells [24].

### 7.3 Akt regulation by anchoring proteins

Several non-substrate proteins have been shown to bind Akt and regulate its activity. The carboxyl-terminal modulator protein (CTMP) interacts with the carboxyl-terminal of Akt at the plasma membrane and reduces its phosphorylation on both Thr308 and Ser473 [25]. Another protein, Trb3, binds to the central kinase region of Akt and also reduces its phosphorylation [26]. The cytoskeletal protein Keratin K10 binds Akt and sequesters it to the cytoskeleton thus inhibiting its ability to translocate to the plasma membrane [27]. Other proteins, such as heat shock proteins 90 [28] and 27 [29] have been demonstrated to bind Akt and positively regulate its activity.
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Chapter 8

Effects of O-GlcNAc increasing agents on Akt

8.0 Introduction

The ubiquitous serine threonine kinase Akt (protein kinase B) is a member of the ACG superfamily of kinases and plays an important role in mediating a variety of cellular functions, particularly in the brain. Akt has been shown to regulate such critical brain processes as the differentiation of neural stem cells into astrocytes [1], regulation of neuronal cell survival [2], and protection against ischemic injury [3]. The misregulation of Akt in the brain has been implicated in the progression of brain cancers from anaplastic astrocytoma to glioblastoma multiforme [4] underscoring the need to fully understand the regulation of this enzyme. Recently, in adipocytes, increased intracellular levels of the O-GlcNAc posttranslational modification have been demonstrated to prevent the insulin-stimulated activation of Akt [5,6]. These recent studies suggest an important new regulatory mechanism for Akt, however, the effects of O-GlcNAc on Akt activation in the brain have yet to be investigated.

Because the O-GlcNAc modification has been shown to affect Akt activation in adipocytes [7,8] and the translocation of other members of the ACG superfamily of kinases in glial cells [9], we sought to determine if increased O-GlcNAc levels affected Akt activity and/or subcellular localization in an SV-40 transformed human glial cell line.

8.1 Materials and methods
8.1.1 Materials

SVG cell line, eagle’s minimum essential medium (EMEM) and fetal bovine serum (FBS) were obtained from American Type Culture Collection (Rockville, MD). Penicillin/streptomycin was purchased from Fisher Scientific (Suwanee, GA). Anti-Akt (catalog #9272) and anti-phospho-Akt (Serine 473) (catalog #4058) specific antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-GRP 78 (catalog # ac-13968) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) whereas the CTD110.6 anti-O-GlcNAc antibody was a kind gift from Dr. Gerald Hart at Johns Hopkins University (Baltimore, MD) and is also available from Covance Research Products (Berkeley, CA). Goat anti-mouse-HRP and goat anti-rabbit-HRP secondary antibodies were from BioRad (Hercules, CA). D-glucosamine, D-galactosamine, N-acetyl-L-cysteine, and streptozotocin (STZ) were purchased from Sigma (St. Louis, MO). O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc) was from Carbogen (Aarau, Switzerland), and 1,2-dideoxy-2'-propyl-α-D-glucopyranoso-[2,1-d]-Δ2'-thiazoline (NAGBT) was kindly provided by Dr. David J. Vocadlo at Simon Fraser University in British Columbia, Canada. All other chemicals were purchased from Sigma and were of the purest grade available.

8.1.2 Cell culture

SVG cells were grown at 37°C in a 95% air and 5% CO₂ environment. Cells were cultured in T-150cm² flask to 90% confluency in EMEM (5.4mM glucose) supplemented with 10% FBS and 10U/mL penicillin and 10µg/mL streptomycin. Cell media was supplemented with 8mM glucosamine, 8mM galactosamine, 5mM STZ, 75µM NAGBT, or 80µM PUGNAc for the time periods indicated. For studies examining the possible
effect of oxidative stress on glucosamine or STZ induced Akt phosphorylation, cells were treated with 6mM N-acetyl-L-cysteine (pH’d to 7.0 in phosphate buffered saline) alone or in combination with 8mM glucosamine or 5mM STZ for 1, 3, or 5 hours.

8.1.3 Cell harvesting and fractionation

At the indicated time points, experiments were terminated by immediately placing flasks of cells were on ice and washing with ice cold phosphate buffered saline (PBS). Cells were scraped into PBS and centrifuged a 2,000xg for 3 minutes to pellet cells. For preparation of whole cell homogenates, PBS was removed and cells were resuspended in 600µl of ice-cold homogenization buffer (20mM Tris, 1mM EGTA, 1mM EDTA, 2mM MgCl₂, 150mM NaCl, 1mM dithiothreitol, 1mM PMSF, 1mM Na₃VO₄, 4µg/ml aprotinin, and 1.0% Triton X-100). Cells were lysed by sonication (Fisher Scientific Sonic Dismembrator F60) on ice with 2, 10-second pulses at 7 watts. Any whole cells or debris was pelleted by centrifuging at 1000xg for 5 minutes at 4°C. Protein concentrations of supernatants were determined using BioRad Protein Assay Dye Reagent using bovine serum albumin as the standard according to the manufacture’s instructions.

Fractionation of cells into cytosol and membrane fractions was done according to a method previously demonstrated to successfully separate membrane associated Akt from cytosolic Akt [10] with few modifications. Briefly, SVG cells were harvest as described above except that cells were initially resuspend in ice-cold homogenization buffer without Triton X-100. After lysing cells by sonication, debris was pelleted by centrifuging at 1000xg for 5 minutes at 4°C. Supernatants were then centrifuged at 100,000xg for 1 hour at 4°C. The resulting supernatants were removed and labeled as
cytosol fractions. Pellets were resuspended in homogenization buffer with 1.0% Triton X-100 by gentle agitation for 30 minutes followed by a brief 2-second sonication at 7 watts. Samples were centrifuged at 100,000xg for 30 minutes at 4°C. Supernatants were removed and labeled as membrane fractions. Protein concentrations of both fractions were determined using BioRad Protein Assay Dye Reagent using bovine serum albumin as the standard according to the manufacture’s instructions.

8.1.4 Electrophoresis and Western blotting

Proteins from cytosol and membrane fractions were mixed with 0.3 volumes of 3x sample buffer (0.18M Tris-HCl pH 6.8, 6% sodium dodecyl sulfate (SDS), 30% glycerol, 0.025% Bromophenol Blue) and equal amounts of protein were loaded onto 8% SDS-polyacrylamide gels. Samples were electrophoresed for 1 hour 30 minutes at 15mAmps and then transferred to nitrocellulose membranes by electroblotting in 50mM Tris, 77mM glycine, and 20% methanol transfer buffer for one hour at 12 volts. For western blot analysis with Akt and phospho-Akt antibodies, membranes were blocked with 5% (w/v) non-fat dried milk in tris buffered saline (140mM NaCl, 2.7mM KCl, and 25mM Tris pH 8.0) with 0.05% Tween-20 (TBS-T). Membranes were then incubated overnight at 4°C in TBS-T with 3% bovine serum albumin (BSA) containing the Akt antibody. For western blots with cytosol fractions, both Akt and phospho-Akt antibodies were diluted 1:2000 while for membrane fractions a dilution of 1:500 was used. Membranes were subsequently washed for 10 minutes (3 times) in TBS-T. Goat anti rabbit-HRP (1:6000 dilution) in TBS-T with 3% BSA was then incubated with the membranes. Membranes were again washed 10 minutes (4 times) in TBS-T and bands were detected by chemiluminescence according to the manufacture’s instructions (Pierce, Rockford, IL).
For analysis using CTD110.6 antibody, membranes were blocked in tris buffered saline with 0.3% Tween-20 (TBS-HT) for 1 hour then incubated overnight at 4°C in TBS-HT containing the CTD110.6 antibody (1:5000 dilution) [11]. Membranes were washed for 10 minutes (2 times) in tris buffered saline with 1.0% Triton X-100, 0.1% SDS, 0.25% deoxycholic acid (TBS-D) and (3 times) in TBS-HT. Goat anti rabbit Ig-M-HRP (1:15,000 dilution) in TBS-HT was then added to membranes. Membranes were washed as before and bands detected using chemiluminescence. Immunoblots were quantified using Scion Image 4.02 analysis program (Scion Corp., Frederick, MD).

8.1.5 Statistical Analysis

Data are given as ± standard error of the mean (S.E.M) for three to five experiments. Comparisons between means were performed using two-tailed Student’s t test for unpaired data and graphed using SigmaPlot 8.0™. Values with p<0.05 were considered significant.

8.2. Results

8.2.1 Effects of glucosamine on Akt phosphorylation and Akt distribution between cytosol and membrane

In order to examine if increased global levels of the O-GlcNAc protein modification affected Akt activity or subcellular distribution, SVG cells were first treated with either 8mM glucosamine, 5mM STZ, 80µM PUGNAc or 75µM NAGBT. Treated cells were then fractionated into cytosol and membrane fractions, separated by SDS-PAGE, immunoblotted, and probed with antibodies against anti-phospho-Akt (Serine 473) or for total Akt (see section 8.1).
Following 8mM glucosamine treatment, phospho-Akt levels in both cytosol and membrane fractions were rapidly and significantly increased when compared to untreated controls. After one hour of treatment, phospho-Akt levels had increased by 43.4% ± 23.6 in cytosol and by 31.3% ± 23.6 in membrane fractions (Figure 8.1). Phospho-Akt levels continued increasing through 3 hours of treatment with increases of 75.7% ± 31.9 in the cytosol and 49.5% ± 41.3 in membrane fractions (Figure 8.1) and were maximal after 5 hours, increasing by 96.8% ± 24.6 in the cytosol and 57.7% ± 38.1 in membrane fractions. After 9 hours of treatment, phospho-Akt levels began to decrease in the cytosol (67.8% ± 18.6) and membrane (33.8% ± 11.4) fractions (Figure 8.1). While phospho-Akt levels were significantly altered following glucosamine treatment, there were no significant changes in total Akt levels in either the cytosol or membrane fraction (Figure 8.1).

8.2.2 Effects of streptozotocin on Akt phosphorylation and Akt distribution between cytosol and membrane

To further investigate the potential role of the O-GlcNAc modification on the activity and cellular distribution of Akt, SVG cells were treated with the O-GlcNAcase inhibitor STZ [12-14] and prepared as describe following glucosamine treatment. Analysis of Akt phosphorylation following 5mM STZ again showed a marked increase in phospho-Akt similar to that seen following glucosamine treatment. After one hour of treatment with 5mM STZ, phospho-Akt levels increased by 44.4% ± 12.3 in cytosol and by 32.3% ± 11.3 in membrane fractions (Figure 8.2). Phospho-Akt levels were maximal after 3 hours of treatment with increases of 96.8% ± 11.0 in the cytosol and 66.3% ± 18.7
Figure 8.1 - Effects of Glucosamine on phospho-Akt and Akt in cytosol and membrane fractions. SVG cells were treated with 8mM glucosamine for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Cytosol and membrane fractions were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt). Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for cytosol and membrane fractions probed with anti-phospho serine-473 Akt or total Akt (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01.
Figure 8.2 - Effects of STZ on phospho-Akt and Akt in cytosol and membrane fractions. SVG cells were treated with 5mM STZ for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Cytosol and membrane fractions were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt). Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for cytosol and membrane fractions probed with anti-phospho serine-473 Akt or total Akt (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01.
in membrane fractions (Figure 8.2). Phospho-Akt levels returned to control levels after 5 hours of treatment in membrane fractions and 9 hours in cytosol fractions (Figure 8.2). Total Akt levels were also analyzed with and without STZ treatment and showed no significant changes from control levels (Figure 8.2).

8.2.3 Effects of PUGNAc and NAGBT on Akt phosphorylation and Akt distribution between cytosol and membrane

In order to determine if the increased Akt phosphorylation observed following cellular treatment with glucosamine and STZ is a result on increased O-GlcNAc protein modification, SVG cells were treated with either 80µM PUGNAc or 75µM NAGBT for 1, 3, 5, or 9 hours and prepared as described for glucosamine treatment. Interestingly, the results obtained from PUGNAc and NAGBT treatment of SVG cells differed from both glucosamine treatment and STZ treatment. Neither 80µM PUGNAc nor 75µM NAGBT incubation resulted in no significant increase in Akt phosphorylation in either the cytosol or membrane fractions at any of the time points examined when compared to untreated controls (Figure 8.3 and 8.4). To determine if exposure of SVG cells to NAGBT for time periods shorter than 1 hour had an effect on phospho-Akt levels, cells were treated for 15, 30, or 45 minutes. These exposures did not significantly alter Akt phosphorylation (data not shown). Also, total levels of Akt in neither the cytosol nor the membrane fractions were significantly altered by these treatments at any time points (Figure 8.3 and 8.4).

Analysis of global O-GlcNAc levels from both cytosol and membrane fractions of SVG cells treated with either glucosamine, STZ, PUGNAc, or NAGBT revealed that, whereas all compounds effectively increased O-GlcNAc levels, glucosamine and STZ treatment resulted in much lower levels of increase. It is therefore possible that the much
Figure 8.3 - Effects of PUGNAc on phospho-Akt and Akt in cytosol and membrane fractions. SVG cells were treated with 80μM PUGNAc for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18μg) were separated by SDS-PAGE, and transferred. Cytosol and membrane fractions were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt). Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for cytosol and membrane fractions probed with anti-phospho serine-473 Akt or total Akt (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01
Figure 8.4 - Effects of NAGBT on phospho-Akt and Akt in cytosol and membrane fractions. SVG cells were treated with 75µM NAGBT for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Samples were separated into cytosol and membrane fractions as described in section 4.1.3. Equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Cytosol and membrane fractions were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt). Immunoblots were analyzed by densitometry and the results were graphed (A). Representative immunoblots for cytosol and membrane fractions probed with anti-phospho serine-473 Akt or total Akt (B). Values are means ± S.E.M. for 5 determinations. * represents p<0.05 and ** represents p<0.01
larger O-GlcNAc increases may inhibit the pathway(s) responsible for the glucosamine and STZ induced increase in Akt phosphorylation. In order to determine if the global level of O-GlcNAc accounted for the different effects of these O-GlcNAc modulating agents, cells were treated with reduced concentrations of PUGNAc and NAGBT. Cells were treated with either 40 µM, 20 µM, or 10 µM NAGBT or PUGNAc for 1 or 3 hours; however, none of these treatment conditions resulted in any detectable increase in Akt phosphorylation (Figure 8.5 and 8.6).

8.2.4 Effects of galactosamine on Akt phosphorylation

Because Akt activation has been demonstrated to be affected by increases in osmotic pressure [15-17], SVG cells were treated with 8mM galactosamine to examine if the concentrations of glucosamine and STZ used in this study affected Akt phosphorylation via an osmotic pressure related pathway. Additionally, the results obtained following treatment with galactosamine would determine if the effects of glucosamine and STZ treatment on Akt are mimicked by other structurally related sugars. Unlike glucosamine and STZ treatment, however, cellular treatment with 8mM galactosamine for 1, 3, 5, or 9 hours did not alter phospho-Akt levels or total Akt levels significantly from untreated control levels (Figure 8.7).

8.2.5 Effects of N-acetyl-L-cysteine on Akt phosphorylation

In addition to osmotic stress, oxidative stress has also been shown to affect Akt activation and glucosamine and STZ have been demonstrated to increase oxidative stress [18]. To examine if the increases in Akt phosphorylation following glucosamine and STZ treatment are a result of oxidative stress, SVG cells were treated with either 8mM glucosamine and 6mM N-acetyl-L-cysteine, 5mM STZ and 6mM N-acetyl-L-cysteine,
Effects of decreasing NAGBT concentrations on phospho-Akt and Akt

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Figure 8.5 - Effects of decreasing NAGBT concentrations on phospho-Akt and Akt. SVG cells were treated with 10µM NAGBT for 1 or 3 hours (10µM T=1 and 10µM T=3 respectively), 20µM NAGBT for 1 or 3 hours (20µM T=1 and 20µM T=3 respectively), or 40µM NAGBT for 1 or 3 hours (40µM T=1 and 40µM T=3 respectively). Additionally, two untreated (Control) samples were prepared. Whole cell homogenates were prepared and equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Immunoblots were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt).
Effects of decreasing PUGNAc concentrations on phospho-Akt and Akt

**Figure 8.6 - Effects of decreasing PUGNAc concentrations on phospho-Akt and Akt.** SVG cells were treated with 10μM PUGNAc for 1 or 3 hours (10μM T=1 and 10μM T=3 respectively), 20μM PUGNAc for 1 or 3 hours (20μM T=1 and 20μM T=3 respectively), or 40μM PUGNAc for 1 or 3 hours (40μM T=1 and 40μM T=3 respectively). Additionally, two untreated (Control) samples were prepared. Whole cell homogenates were prepared and equal amounts of protein (18μg) were separated by SDS-PAGE, and transferred. Immunoblots were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt).
Figure 8.7 - **Effects of Galactosamine on phospho-Akt and Akt.** SVG cells were treated with 8mM galactosamine for 1 hour (T=1), 3 hours (T=3), 5 hours (T=5), 9 hours (T=9). Untreated (control) samples were prepared for each experiment. Whole cell homogenates were prepared and equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Immunoblots were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt).
6mM N-acetyl-L-cysteine alone, or untreated for 1, 3, or 5 hours. Cellular treatment with N-acetyl-L-cysteine has been well documented to attenuate the effects of oxidative stress in other systems [18]. In our system 6mM N-acetyl-L-cysteine treatment did not significantly alter the increased phospho-Akt levels observed following 8mM glucosamine or 5mM STZ alone (Figure 8.8 and 8.9). Furthermore, 6mM N-acetyl-L-cysteine treatment did not alter phospho-Akt or total Akt levels from untreated control levels at any of the time points analyzed (Figure 8.8 and 8.9).

8.2.6 Effects of Glucosamine or STZ on GRP 78 expression

GRP 78 is a member of the heat shock protein family whose expression has been shown to increase in response to endoplasmic reticulum (ER) stress [31, 34]. In order to determine if either glucosamine or STZ were inducing ER stress, thus giving a possible mechanism for the increases in phospho-Akt, cells were treated with either 8mM glucosamine or 5mM STZ and samples were western blotted for GRP 78. Results indicated an increase in GRP 78 expression after 5 hours of glucosamine treatment followed by a much larger increase 9 hours post treatment. Cells treated with 5mM STZ showed no increase in GRP 78 expression at any of the time points analyzed.

8.3 Discussion

The O-GlcNAc modification has been shown to regulate the activity of key signal transduction enzymes including PI 3-K in endothelial cells [19], MAPK in neutrophils [20], eNOS in rat penis tissue [21] and Akt in adipocytes [7,8]. Previously, our research in human glial cells, suggests that increased levels of this modification also decreases the active, membrane bound forms of PKC-ε and possibly PKC-α (chapter 4), two kinases that share a high degree of structural and sequence homology with Akt [22]. In order to
Effects of N-Acetylcysteine and Glucosamine on Akt

Figure 8.8 - Effects of N-acetylcysteine and Glucosamine on phospho-Akt and Akt.
SVG cells were treated with either 8mM glucosamine and 6mM N-acetylcysteine for 1, 3, or 5 hours (GlcN+NAC T=1, GlcN+NAC T=3, GlcN+NAC T=5 respectively) or 6mM N-acetylcysteine alone for 1, 3, or 5 hours (NAC T=1, NAC T=3, NAC T=5 respectively). Additionally, two untreated (Control) samples were prepared. Whole cell homogenates were prepared and equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Immunoblots were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt).
Figure 8.9 - Effects of N-acetylcysteine and STZ on phospho-Akt and Akt. SVG cells were treated with either 5mM STZ and 6mM N-acetylcysteine for 1, 3, or 5 hours (GlcN+NAC T=1, GlcN+NAC T=3, GlcN+NAC T=5 respectively) or 6mM N-acetylcysteine alone for 1, 3, or 5 hours (NAC T=1, NAC T=3, NAC T=5 respectively). Additionally, two untreated (Control) samples were prepared. Whole cell homogenates were prepared and equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Immunoblots were treated with either phospho-specific Akt antibodies (p-Akt) or antibodies against total Akt (Akt).
Effects of Glucosamine or STZ on GRP 78 expression

Figure 8.10 - Effects of Glucosamine or STZ on GRP 78 expression. SVG cells were treated with either 8mM glucosamine (top, GlcN) or 5mM STZ (bottom, STZ) for 1, 3, 5 or 9 hours (T=1, T=3, T=5, or T=9 respectively). Additionally, four untreated (Control) samples were prepared. Whole cell homogenates were prepared and equal amounts of protein (18µg) were separated by SDS-PAGE, and transferred. Immunoblots were treated with antibodies against total GRP 78.
further investigate the effects of the O-GlcNAc protein modification on signal transduction pathways in the brain, we sought to examine if Akt, like PKC-ε and possibly PKC-α, either the activity or subcellular localization affected by increases in this modification in glial cells. As indicated by our results, cellular treatment with four separate O-GlcNAc modulating agents produced both different levels of global O-GlcNAc increase as well as different effects on Akt phosphorylation. Glucosamine and STZ treatments produced low-level sustained increases in global O-GlcNAc (Figure 4.1 and 4.2) and also a rapid rise in phospho-Akt levels in both cytosol and membrane fractions. These increases reached identical maxima that were followed by a steady decline toward basal levels. The similarities between the glucosamine and STZ induced increases in Akt phosphorylation suggest that both compounds activate Akt via the same pathway. Alternatively, the more potent O-GlcNAcase inhibitors PUGNAc and NAGBT produced relatively large, rapidly rising O-GlcNAc increases (Figure 4.1 and 4.2) but failed to significantly alter phospho-Akt levels from basal levels.

One possible explanation for the data is that the low level, global increases in O-GlcNAc correspond to the modification of one or more specific proteins that then trigger an Akt activation pathway. As total levels of the O-GlcNAc modification continue to accumulate additional proteins are modified (or the same proteins increasingly modified) reaching a level sufficient to stimulate a deactivation pathway and/or block the activation pathway for Akt. Cellular treatment with lower concentrations of PUGNAc and NAGBT, however, failed to activate Akt suggesting that the effects of glucosamine and STZ are not due to increases in the O-GlcNAc modification.
Additionally, the increases in O-GlcNAc do not appear to affect Akt transcription, degradation, or translocation since neither treatment altered total Akt levels or its intracellular distribution between the cytosol and membrane (although a change in Akt transcription and a proportional, opposite change in Akt degradation cannot be ruled out). Similarly, incubation of adipocytes for 18 hours with 2.5mM glucosamine has been shown not to affect the translocation of Akt to the plasma membrane [23] suggesting that O-GlcNAc does not regulate Akt translocation in insulin independent or dependent cell types.

Another possible explanation for the effects of glucosamine and STZ on Akt is that the experimental concentrations used (8mM and 5mM respectively) increased osmotic stress. Akt has been shown to be activated [17] by hyperosmolarity in renal tubular cells. Treatment of cells with 8mM galactosamine, an equimolar osmotic alteration that does not enhance the O-GlcNAc modification however, did not result in significant alterations in Akt phosphorylation. This data indicates that the increased Akt phosphorylation following 8mM glucosamine and 5mM STZ treatments is a response to hyperosmotic conditions. Furthermore, the lack of effect of galactosamine on phospho-Akt levels also indicates that the effects of glucosamine and STZ are not mimicked by another structurally similar sugar.

An alternate hypothesis explaining the effects of glucosamine and STZ on Akt phosphorylation, is Akt activation due to increased oxidative stress. Akt has been shown to become activated under conditions of oxidative stress [24,25]. Furthermore, in addition to modulating intracellular O-GlcNAc levels, cellular treatment with glucosamine [18] or STZ [26,27] is known to increase oxidative stress. Increased flux
through the hexosamine biosynthetic pathway as a result of glucosamine treatment has been shown to lead to pancreatic β-cell deterioration presumably as a result of oxidative stress not related to increases in the O-GlcNAc modification [18]. Although the exact mechanism by which glucosamine produces oxidative stress is unknown, it has been shown to increase H₂O₂ levels [18]. Additionally, long term exposure to STZ has been shown to increase nitric oxide levels and suppress glutathione peroxidase activity in STZ treated rat brain [26]. Many of the effects of glucosamine [18] and STZ induced oxidative stress, such as reduced arterial blood pressure [28], have been demonstrated to be reduced or reversed by N-acetylcysteine treatment. N-acetylcysteine is readily reduced to cysteine and can thus increase intracellular levels of reduced glutathione [29,30]. The increases in phospho-Akt levels seen following exposure to glucosamine and STZ were not attenuated by treatment with N-acetylcysteine indicating that the mechanism of Akt activation does not likely involve oxidative stress.

In addition to O-GlcNAc protein modification, oxidative stress, and osmotic stress, glucosamine has been shown to affect several other cellular functions. Glucosamine has been shown to induce endoplasmic reticulum (ER) stress in several cell systems [31,32]. The effects of STZ on ER stress, however, have not been examined. ER stress is described as a disruption of ER homeostasis that interferes with normal protein folding leading to an accumulation of misfolded or unfolded proteins [33]. Akt has been demonstrated to be activated in MCF-7 human breast cancer cells and H1299 human lung cancer cells in response to thapsigargin and tunicamycin induced ER stress [34]. Similar to our pattern of Akt activation following glucosamine and STZ treatment, the pattern of Akt activation observed by Hu et al. [34] occurred rapidly with maximal
levels occurring around 4 hours post treatment and then returning to basal levels between 8 and 12 hours post treatment.

Analysis of GRP 78 expression, a well documented indicator of ER stress [31, 34], indicated that glucosamine, but not STZ, was inducing ER stress in our astroglial cell model. These results suggest that the increases in phospho-Akt induced glucosamine are due to increased ER stress whereas the increases in phospho-Akt observed after STZ treatment may be mediated via a different mechanism. STZ is a well known nitrosylating agent and may be inducing cellular stress via DNA damage.

Although the mechanism by which glucosamine may induce ER stress in not known, these compounds may disrupt normal protein N-glycosylation via the accumulation of lipid-linked oligosaccharide (LLO) such as Glc₃Man₉GlcNAc₂-P-P-dolichol that have been shown to induce ER stress [35]. Additionally, glucosamine may disrupt the normal activity of N-acetylglucosaminyltransferase V, an enzyme found predominately in intestine, lung and brain [36,37] that facilitates ER stress when its activity is blocked [38].

Although increased ER stress has been shown to activate Akt, the mechanism by which this activation occurs is not known [34]. Two potential mechanisms by which ER stress may activate Akt are via a disruption on Ca²⁺ homeostasis or the activation of stress activated protein kinases. Ca²⁺ and calmodulin are known to activate PI 3-K, a well known upstream activator of Akt. Our earlier findings show that whereas 8mM glucosamine treatment also resulted in an activation of the Ca²⁺ sensitive PKC-βII similar to that of Akt, it failed to activate the Ca²⁺ sensitive PKC-α [9]. Furthermore, Kohout et al. [39] demonstrated that PKC-α binds to the plasma membrane longer and more
efficiently at lower intracellular Ca\(^{2+}\) concentrations than PKC-βII suggesting that increases in Ca\(^{2+}\) likely do not account for our results. Another possible mechanism by which ER stress could activate Akt is by the activation of the protein kinase R-like endoplasmic reticulum associated protein kinase (PERK) or the high inositol requiring-1 protein kinase (IRE1). Both of the ER transmembrane proteins are Ser/Thr kinases that are activated in response to ER stress [40]. Recently PERK has been demonstrated to be rapidly activated by 3mM glucosamine in retinal neuronal cells [40] and attributed this activation to ER stress. Although there is no evidence that PERK or IRE1 directly phosphorylate Akt, they are known to activate a variety of signal transduction pathways [41].

In conclusion, this study demonstrates a novel activation of Akt in response to glucosamine and STZ treatments. These treatments do not appear to affect the translation, degradation, or translocation of the enzyme. Investigation of the cause of this activation suggests that it is not related to increases in the O-GlcNAc protein modification, increased osmotic stress, or oxidative stress. It is likely that the increase in phospho-Akt brought on by glucosamine treatment is a result of increased ER stress and the increases observed after STZ treatment are mediated via an alternate mechanism.
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Chapter 9

PKC-ε and PKC-α associated proteins

9.0 Introduction

PKC associated proteins have been shown to play a role in PKC isoform activation, translocation, and localization near the proper substrates and regulators [1-4] (discussed in section 2.3). Additionally, the O-GlcNAc protein modification has been shown to regulate protein-protein interaction in certain instances [5-7] (discussed in section 1.4.3). It is therefore possible that the decreases in membrane associated PKC-ε and -α following treatment with O-GlcNAc increasing compounds (discussed in chapter 4) is a result of a disruption between these PKC isoforms and certain O-GlcNAc modified protein(s) that play a role in PKC-ε and -α membrane association. The goal of this chapter is to determine if any O-GlcNAc modified proteins specifically associate with either PKC-ε or -α thus opening new avenues for the investigation of the possible link between PKC regulation and the O-GlcNAc modification. Additionally, we sought to examine if there is an association between either of these isoforms and the related kinase Akt.

9.1 Materials and Methods

Cell culturing, SDS-PAGE, and immunoblotting were carried out as described in section 4.1. Immunoprecipitations were done as described in section 5.3.2.

9.2 Results
9.2.1 O-GlcNAc modified proteins associated with PKC-ε and -α

Untreated SVG cells were immunoprecipitated with either anti-PKC-ε or anti PKC-α antibodies or the appropriate control antibody (see section 5.3.2). Additionally, the immunoprecipitation process was carried out using Protein A agarose beads with no antibody in order to be able to subtract the proteins binding nonspecifically to the beads from those specifically binding to the antibodies. Samples were then immunoblotted using the CTD110.6 anti-O-GlcNAc antibody in order to detect O-GlcNAc modified proteins that were immunoprecipitated. Comparison of anti-O-GlcNAc immunoblots of samples immunoprecipitated with either anti-PKC-ε and -α antibodies, control antibodies, or beads only revealed three distinct protein bands specifically precipitated with the anti-PKC-ε and -α antibodies. One O-GlcNAc modified protein of ~120kDa was immunoprecipitated by the anti-PKC-ε antibody and two O-GlcNAc modified proteins of ~90kDa and ~80kDa were brought down with PKC-α (Figure 9.1).

9.2.2 PKC-ε and PKC-α association with Akt

In order to determine if either PKC-ε or -α associated with Akt, immunoprecipitations were performed using either PKC-ε or PKC-α specific antibodies followed by immunoblotting for Akt. Some immunoprecipitations were performed using IP buffer (see section 5.3.2) with either 1.0% or 0.1% Triton X-100 as detergent concentration has been shown to affect the association of Akt with certain proteins in immunoprecipitation experiments [8]. Immunoprecipitation with anti-PKC-ε antibodies followed by immunoblotting for Akt revealed no significant association between the two
Association between PKC-ε and -α and O-GlcNAc modified proteins

Figure 9.1 – Association between PKC-ε and -α and O-GlcNAc modified proteins.

Untreated SVG cells were lysed and immunoprecipitated with anti-PKC-ε antibodies (IP PKC-ε), anti-PKC-α antibodies (IP PKC-α), normal rabbit IgG antibodies (IP rabbit IgG), normal mouse IgG (IP mouse IgG), or Protein A agarose beads with no antibodies (beads). Samples were separated by SDS-PAGE and immunoblotted using the CTD110.6 anti-O-GlcNAc antibody. * indicates bands unique to immunoprecipitations performed using PKC-ε or -α specific antibodies and not found in control immunoprecipitations.
Figure 9.2 – Association between PKC-ε and Akt.
Untreated SVG cells were lysed and immunoprecipitated with anti-PKC-ε antibodies (IP PKC-ε) or normal rabbit IgG antibodies (IP control IgG) in IP buffer containing either 1.0% or 0.1% Triton X-100. Samples were separated by SDS-PAGE and immunoblotted using anti-Akt antibodies. Non-immunoprecipitated whole cell homogenate (homogenate) was also analyzed.
Figure 9.3 – Association between PKC-α and Akt.

Untreated SVG cells were lysed and immunoprecipitated with anti-PKC-α antibodies (IP PKC-ε), normal mouse IgG antibodies (IP mouse IgG1), anti-p27 antibodies, anti-Mat1 antibodies, or Protein A agarose beads with no antibodies. Samples were separated by SDS-PAGE and immunoblotted using anti-Akt antibodies. Non-immunoprecipitated whole cell homogenate (homogenate) was also analyzed.
proteins when compared to immunoprecipitation with control antibodies using either 1.0% or 0.1% Triton X-100 in the IP buffer (Figure 9.2).

Investigation of a possible association between PKC-α and Akt was done by immunoprecipitating PKC-α from untreated SVG cells with an anti-PKC-α mouse monoclonal antibody (Santa Cruz Biotechnology)(see section 5.4.2). Control immunoprecipitations were performed using normal mouse IgG, anti-p27 and anti-Mat1 mouse monoclonal antibodies (all from Santa Cruz Biotechnology), and Protein A agarose beads (discussed further in section 5.4.2). Immunoprecipitation with anti-PKC-α antibodies followed by immunoblotting for Akt revealed a significant association between PKC-α and Akt when compared to control immunoprecipitations or using Protein A agarose beads only (Figure 9.3).

9.3 Discussion

PKC associated proteins have been shown to play a role in its subcellular localization and its ability to translocate to the cell membrane upon activation [1-4]. There is no record in the literature, however, of analysis of any of these proteins for modification with O-GlcNAc. The finding that specific proteins associate with PKC-ε and PKC-α in human astroglial cells raises the possibility that the decreases in membrane associated PKC-ε and -α observed following treatment with O-GlcNAc modulating agents may be mediated through their interaction with these modified proteins. Further analysis, including purification, identification, and characterization of these O-GlcNAc modified proteins, is required in order to determine their role, if any, in the regulation of PKC-ε and -α.
Additionally, the finding that PKC-α associates with Akt in human astroglial cells raises some interesting possibilities. This association may indicate cross-talk between the PKC-α and Akt mediated signal transduction pathways in astroglial cells. Further investigation is needed to reveal the significance of this interaction.
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About the Author

Jason Aaron Matthews was born on June 17, 1971 in Waycross, GA. Following elementary school he and his family moved to Birmingham, AL where he completed junior high and high school. After graduating salutatorian of his class, he moved to Jacksonville, FL and attended the University of North Florida. He graduated in 1994 with a major in Biology. A few years later, he then moved to Tampa, FL and entered the graduate program in the department of chemistry at the University of South Florida. He joined the laboratory of Dr. Robert Potter, a biochemistry professor, where he worked until obtaining his Ph.D. degree.