Stimulation of Glucose Transport: Potential Role of Akt1/Protein Kinase Bα

by

Elena Bogdanovic

A thesis submitted in conformity with the requirements for the degree of Master of Science, Graduate Department of Physiology, University of Toronto

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ABSTRACT

Akt1/Protein Kinase Bα (Akt1/PKBα) has been shown to lie downstream of phosphatidylinositol 3-kinase (PI3K) in the insulin action pathway and has been implicated in the stimulation of glucose transport and Glut4 translocation by insulin. To obtain further insight into this role of Akt1/PKBα, two models were used.

Vanadate and pervanadate (pV) are protein tyrosine phosphatase inhibitors that can stimulate glucose uptake and Glut4 translocation in L6 myotubes independently of PI3K. The present study showed that while insulin and pV increase Akt1/PKBα activity in a PI3K dependent manner, vanadate did not. These data suggest that Akt1/PKBα is important for insulin but not for vanadate or pV stimulated glucose uptake.

The second model examined Akt1/PKBα activation in insulin resistant rat adipocytes. Rat adipocytes rendered insulin resistant by chronic exposure to hyperglycemia and hyperinsulinemia (High G/l) showed decreased insulin-stimulated glucose transport and Glut4 translocation and Akt1/PKBα activation. This was associated with normal IRS-1 tyrosine phosphorylation and IRS-1 associated p85. Co-incubation with NAC (N-acetyl-cysteine) prevented the insulin resistance in glucose uptake, Glut4 translocation and Akt1/PKBα.

Taken together, these findings suggest a role for Akt1/PKBα to mediate insulin-stimulated glucose transport. However, additional pathways must also exist that can promote glucose uptake and Glut4 translocation.
ACKNOWLEDGEMENTS

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<th>Definition</th>
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<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
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</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GFA</td>
<td>Glutamine: fructose-6-phosphate amidotransferase</td>
</tr>
<tr>
<td>Glc</td>
<td>glucosamine</td>
</tr>
<tr>
<td>GLDH</td>
<td>L-glutamate dehydrogenase</td>
</tr>
<tr>
<td>Glut</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>HBP</td>
<td>hexosamine biosynthesis pathway</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
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<tr>
<td>HDM</td>
<td>high-density microsome</td>
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<tr>
<td>HEPES</td>
<td>N-[2-Hydroxyethyl]piperazine-N'[2-ethanesulfonic acid]</td>
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<tr>
<td>High G/I</td>
<td>combined high glucose and high insulin concentrations</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>KRBH</td>
<td>Kreb's Ringer Bicarbonate HEPES buffer</td>
</tr>
<tr>
<td>LDM</td>
<td>low-density microsome</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAPKAP</td>
<td>mitogen activated protein kinase activated protein</td>
</tr>
<tr>
<td>α-MEM</td>
<td>alpha Minimum Essential Medium</td>
</tr>
<tr>
<td>MOPS</td>
<td>(3-[N-Morpholino]propanesulfonic acid)</td>
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<td>NAC</td>
<td>N-acetyl-cysteine</td>
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<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>NADH</td>
<td>reduced NAD</td>
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<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
</tr>
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<td>NIH</td>
<td>National Institute of Health</td>
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<td>NMR</td>
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<tr>
<td>8-OHdG</td>
<td>8-hydroxydeoxyguanosine</td>
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<td>PDGF</td>
<td>platelet derived growth factor</td>
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<td>PDK</td>
<td>phosphatidylinositol dependent protein kinase</td>
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<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
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<td>PtdIns-3-P</td>
<td>phosphatidylinositol 3-phosphate</td>
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<td>PTP</td>
<td>protein tyrosine phosphatase</td>
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<tr>
<td>pV</td>
<td>pervanadate</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
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<td>SH2</td>
<td>src homology 2</td>
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CHAPTER ONE

INSULIN STIMULATION OF GLUCOSE TRANSPORT
CHAPTER ONE
INSULIN STIMULATION OF GLUCOSE TRANSPORT

Insulin, a peptide hormone, was first discovered by Frederick Banting and Charles Best in 1921 (1). Insulin is an anabolic hormone and the principal regulator of energy metabolism (2). One action of insulin is to promote glucose entry into muscle and adipose tissue (2). Insulin increases glucose entry into these tissues by promoting the translocation of glucose transporters from intracellular low-density microsomes (LDM) to the plasma membrane (PM) (3). Once recruited, the glucose transporters insert into the PM and are likely activated (4) allowing the uptake of glucose into the cell. This action of insulin is particularly important for glucose homeostasis in normal physiology. This is evident in diseases such as Type 2 diabetes in which the decreased ability of insulin to promote glucose uptake in target tissues is a major defect and may contribute to the development of the disease (2,4).

How the activation of the insulin receptor promotes glucose transporter translocation and glucose uptake is not entirely known but several signaling proteins have been implicated.

1.1 Overview of Glucose Uptake Stimulation by Insulin

Full stimulation of glucose transport by insulin requires translocation of glucose transporters (Gluts) from internal membranes to the PM (3), associated with an increase in intrinsic activity of the transporters (4). Insulin stimulates both processes likely by parallel pathways (4), although more is known about the pathway that leads to glucose transporter translocation (Fig. 1.1).
FIG. 1.1 STIMULATION OF GLUT4 TRANSLOCATION BY INSULIN
1.1.1 The Insulin Receptor and IRS Proteins

The insulin receptor contains two α and two β subunits (5). The α subunits are located entirely on the extracellular side of the plasma membrane and contain the insulin binding sites (5). The β subunits are transmembrane proteins that contain tyrosine kinase activity in the cytoplasmic portion (5). Insulin binding to the α subunit leads to the activation of the receptor tyrosine kinase. Once active, the insulin receptor kinase phosphorylates numerous substrates on tyrosine residues which then function as docking proteins (5). There are at least four insulin receptor substrates (IRSs) identified, IRS-1 (6), IRS-2 (7), IRS-3 (8), and IRS-4 (9). Once phosphorylated on tyrosine residues, the IRS proteins recruit and activate Src homology 2 domain (SH2) containing proteins. For the stimulation of glucose transport, the p85 subunit of Class IA phosphatidylinositol 3-kinases appears to be the central protein recruited to the tyrosine phosphorylated IRS proteins (10,11).

1.1.2 Phosphatidylinositol 3-Kinase

Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that catalyze the phosphorylation of phosphoinositides on the 3-position of the inositol ring (12). Four different lipid products of the PI3K family have been identified; PtdIns-3-P, PtdIns-3,4-P$_2$, PtdIns-3,5-P$_2$, and PtdIns-3,4,5-P$_3$ (12). The Class IA PI3K have been demonstrated to play a role in the stimulation of glucose uptake by insulin, most likely by regulating the trafficking of glucose transporter containing vesicles (13). Class IA PI3K are composed of two subunits: the p85 regulatory subunit, and the p110 catalytic subunit (12). The p85
subunit contains two SH2 domains which bind to specific tyrosine phosphorylated motifs on the IRS proteins (10) leading to the activation of the p110 catalytic subunit (14). In vitro, p110 can catalyze the formation of the above mentioned lipids but in vivo only PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ levels are increased by insulin and other extracellular signals (12). PtdIns-3-P is constitutively present inside the cell (12).

1.1.3 Downstream Targets of Phosphatidylinositol 3-Kinase

There are 2 important serine/threonine kinases that have been shown to act downstream of PI3K and may play a role in insulin-stimulated glucose transporter translocation. The first kinase is Akt/PKB (15). There are 3 isoforms discovered to date: Akt1/PKBα (16-18), Akt2/PKBβ (19), and Akt3/PKBγ (20). All isoforms possess an N-terminal pleckstrin homology (PH) domain, followed by a catalytic domain and a C-terminal tail (21). Akt3/PKBγ is truncated at the C terminal compared to the other 2 isoforms (20). Full activation of Akt/PKB requires phosphorylation on threonine (Thr308 on Akt1/PKBα, Thr309 on Akt2/PKBβ, Thr305 on Akt3/PKBγ) and serine residues (Ser473 on Akt1/PKBα, Ser474 on Akt2/PKBβ) (22). Phosphorylation of these residues has been shown to be dependent on PI3K (21). The enzyme which phosphorylates the threonine residue has been purified from rabbit skeletal muscle and shown to be dependent on PtdIns-3,4-P₂ and PtdIns-3,4,5,-P₃ for activation. It was therefore termed phosphoinositide-dependent protein kinase 1 (PDK1) (23). Like Akt/PKB, PDK1 also contains a PH domain (24). The kinase which phosphorylates the serine residue, termed PDK2, has not yet been identified but it will likely be regulated in the same manner as PDK1 and also contain a PH domain (22). The mechanism of
Akt/PKB activation via PI3K is not entirely understood, but a model has been proposed for the activation of Akt/PKB \textit{in vivo}. In this model, PtdIns-3,4-P$_2$ and PtdIns-3,4,5-P$_3$ recruit Akt/PKB to the plasma membrane (24). This is accomplished by the presence of the PH domain in Akt/PKB (24). Once at the PM, PtdIns-3,4-P$_2$ and PtdIns-3,4,5-P$_3$ may promote conformational changes in Akt/PKB which would allow PDK1 and PDK2 to phosphorylate and activate the kinase (12).

The second serine/threonine kinase that has been implicated in the stimulation of glucose transporter translocation by insulin is a group of enzymes known as the atypical PKCs, PKC$\xi$ and PKC$\lambda$ (25). Insulin activation of PKC$\xi$ and PKC$\lambda$ has been shown to be dependent on increased PtdIns-3,4,5-P$_3$, and PDK-1 dependent phosphorylation of activation loop sites in PKC$\xi$ and PKC$\lambda$ (26). Adipocytes transiently transfected with kinase inactive mutants of PKC$\xi$ and PKC$\lambda$ displayed a 40-60% inhibition of insulin-stimulated epitope-tagged Glut translocation (25).

\subsection{1.1.4 Glucose Transporters}

The glucose transporter (Glut) family belongs to a large superfamily of proteins involved in transport of hexoses and other carbon compounds (27). The glucose transporters are transmembrane proteins that transport glucose down its concentration gradient. There are six Glut isoforms: Glut1, Glut2, Glut3, Glut4, Glut5, and Glut7, which are specifically expressed in various tissues and are all products of different genes (27).

Glut4 is the insulin responsive glucose transporter and accounts for the increases in glucose uptake observed with insulin stimulation. It is highly expressed in skeletal
muscle, heart and adipocytes (28). Inside the cell, the majority of Glut4 is contained in the high- and low- density microsomes (29). In the basal state, Glut4 containing vesicles continuously cycle between the low-density microsomes and the plasma membrane (30). Upon stimulation with insulin, the exocytosis of Glut4 vesicles is greatly increased, with a smaller decrease in Glut4 vesicle endocytosis (31). The net result is an increase in the plasma membrane content of Glut4. Glut1 is expressed in all tissues (27). In muscle cells and adipocytes, Glut1 is constitutively present in the plasma membrane (27) and provides the cell with glucose under basal conditions (27).

1.1.5 Intrinsic Activity of Glucose Transporters

Several lines of evidence suggest that insulin stimulates another pathway(s) which leads to increased intrinsic activity of the transporters. First, Katagiri et al. (32) have shown a discrepancy between PI3K activation and glucose transport activity. In this study, a dominant negative p85α regulatory subunit (Δp85α) of PI3K was overexpressed in 3T3-L1 adipocytes. Overexpression of Δp85α markedly inhibited insulin stimulated PI3K activity and glucose transporter translocation, but the inhibition of glucose uptake was less remarkable. In another study (33), the addition of exogenous PtdIns-3,4,5-P₃ lipids to 3T3-L1 adipocytes largely restored hexose uptake blocked by the PI3K inhibitor wortmannin but treatment of basal cells with PtdIns-3,4,5-P₃ alone did not stimulate hexose uptake. Recently, Sweeney et al. (4) have shown that insulin activates the p38 mitogen activated protein (MAP) kinase in 3T3-L1 adipocytes and L6 myotubes. Preincubation of 3T3-L1 adipocytes and L6 myotubes with the p38 MAP kinase inhibitor SB203580 inhibited insulin stimulated glucose uptake but did not affect glucose
transporter translocation. SB203580 had no effect on IRS-1 tyrosine phosphorylation or its association with p85, Akt1/PKBα, Akt2/PKBβ, or Akt3/PKBγ. Together these findings suggest the presence of another signaling pathway(s), separate from the one described above, that involves p38 MAP kinase and results in the increased intrinsic activity of glucose transporters (4).

1.1.6 Subcellular Localization

For insulin to stimulate Glut4 translocation, it appears that the proteins involved in insulin signal transduction must be activated in the correct subcellular fraction (13). For example, platelet-derived growth factor (PDGF) increases PI3K activity in 3T3-L1 adipocytes but does not activate glucose transport (34). This observation is explained by the failure of PDGF to activate PI3K in the LDM (13). Kelly et al. (35) have shown that insulin predominately activates PI3K in the LDM fraction, and to a lesser extent in the PM. IRS-1 and IRS-2, located mainly in the LDM in rat adipocytes, have been implicated in the stimulation of glucose transport by insulin (36), whereas IRS-3 located in the PM was not essential for glucose transport in these cells (37). In addition, the majority (75%) of anti-phosphotyrosine immunoprecipitable PI3K activity was found in the LDM in response to insulin, with only a minor portion found in the PM (35). Cytosolic PI3K activity was not increased in response to insulin, nor was it immunoprecipitable with anti-phosphotyrosine antibodies (35). Together these observations suggest that for insulin to stimulate glucose transport, the subcellular distribution, and not just the presence of the proteins is important (22).
INTRODUCTION TO CHAPTER 2 AND CHAPTER 3

The discovery in 1995 that Akt/PKB can be activated by insulin via PI3K (15) has led to the implication of this enzyme in a variety of insulin stimulated processes (22). One such process is the stimulation of Glut4 translocation by insulin. There are several lines of evidence which support a role for Akt/PKB in the translocation of Glut4. First, Akt/PKB becomes constitutively active upon the addition of a src myristoylation signal which targets the kinase to the membrane (38). Kohn et al. (38) have shown that expression of such a constitutively active Akt/PKB where the PH domain was replaced by the src myristoylation signal stimulated glucose uptake and Glut4 translocation to the same extent as insulin in basal 3T3-L1 adipocytes. Similar results were found using another constitutively active mutant Akt/PKB, Gag-PKB (39,40,41). Gag-PKB is created by fusion of the Gag protein with the PKB N-terminus. This construct corresponds to the constitutively active retroviral protein AKT8 (15). Gag-PKB is also targeted to the membranes (39). Transfection of Gag-PKB into rat adipocytes (39) and L6 muscle cells (41) increased the cell surface content of Glut4 in the absence of insulin. In addition, transfection of Gag-PKB into L6 muscle cells and 3T3-L1 adipocytes increased basal glucose uptake (40). The second line of evidence to support a role for Akt/PKB in the stimulation of Glut4 translocation by insulin came from using the dominant-negative inhibitor AAA-PKB (41), a mutant Akt1/PKBα which has alanine residues substituted at the two phosphorylation sites (Thr308 and Ser473) and at the ATP binding site (Lys179). This mutant is kinase inactive and phosphorylation deficient. Wang et al. (41) have shown that AAA-PKB inhibited the activation of co-transfected wild type and
endogenous Akt/PKB by insulin in L6 muscle cells. AAA-PKB markedly inhibited the increase in Glut4 at the cell surface in response to insulin. In these same cells, co-transfection and overall expression of wild type PKB restored Glut4 translocation. Additionally, Akt2/PKBβ has been shown to co-localize with Glut4 containing vesicles in the basal state and upon insulin stimulation the level of Akt2/PKBβ in the Glut4 vesicles increased, suggesting translocation (29). Kupriyanova et al. (42) also showed that Akt2/PKBβ was recruited to the Glut4 containing vesicles in response to insulin and phosphorylated several vesicle proteins including Glut4. Taken together these studies implicate Akt/PKB in the signal transduction pathway leading to Glut4 translocation in response to insulin.

The hypothesis tested in this thesis are (1) that Akt1/PKBα play a critical role in insulin-stimulated glucose transport and Glut4 translocation, and (2) that this enzyme also plays a role in vanadate and pervanadate stimulated glucose transport. The studies described in Chapter 2 and Chapter 3 use 2 approaches and two different models to test these hypotheses and determine the importance of Akt1/PKBα in the stimulation of Glut4 translocation.

The specific aim of Chapter 2 was to determine the potential role of Akt1/PKBα in vanadate and pervanadate stimulated Glut4 translocation in skeletal muscle. Vanadate and pervanadate (pV) are protein tyrosine phosphatase (PTP) inhibitors which can mimic the action of insulin to stimulate Glut4 translocation and glucose uptake in these cells (43).
In Chapter 3, the activity of Akt1/PKBα was determined in an established model of insulin resistance in which insulin-stimulated glucose transport is defective. The specific aims of Chapter 3 were to:

1) determine if the defect in insulin-stimulated Glut4 translocation found in rat adipocytes rendered insulin resistant by treatment with high glucose and insulin, was associated with a concomitant defect in insulin stimulation of Akt1/PKBα activity,

2) determine whether N-acetyl-cysteine (NAC), an antioxidant and glutathione (GSH) precursor (44), which had previously been found to prevent glucose transport defects in this model, could also prevent any defect in Akt1/PKBα activity,

3) investigate the upstream activators of Akt1/PKBα, namely IRS-1 and IRS-1 associated p85 in cells treated with high glucose/insulin and NAC, to localize the site of the defect in the insulin signaling pathway,

The results from these studies would provide further insight into the role of Akt1/PKBα in the stimulation of Glut4 translocation.
CHAPTER TWO

Role of Akt1/PKBα in Vanadate and Pervanadate Stimulated Glucose Uptake
Vanadate and pervanadate (pV) are protein tyrosine phosphatase inhibitors and insulin mimetic agents. Treatment of L6 myotubes with vanadate and pV increased glucose transport and glucose transporter (Glut) translocation to the same extent as insulin but independently of PI3K. The serine/threonine kinase Akt1/PKBα which lies downstream of PI3K, has been implicated in the stimulation of Glut4 translocation by insulin. Although Akt1/PKBα is activated by insulin in a PI3K sensitive manner, other agents can activate the kinase independently of PI3K. Therefore, the present study examined whether Akt1/PKBα was activated by vanadate and pV in L6 myotubes. Insulin and pV increased the activity of Akt1/PKBα by 2.3±0.4 and 17±2.1 fold above control respectively (p<0.05). We previously found that while pretreatment with the PI3K inhibitor wortmannin inhibited insulin-stimulated glucose transport, it did not inhibit that stimulated by vanadate or pV. However, in the present study wortmannin pretreatment prevented the activation of Akt1/PKBα by insulin (1.08±0.3) and pV (1.8±0.6). On the other hand, vanadate treatment for 30 minutes (1.15±0.18) or 60 minutes (0.9±0.1) did not significantly activate Akt1/PKBα. These results were confirmed by immunoblot analysis of Ser473 phosphorylated Akt1/PKBα. Vanadate and pV stimulate glucose transport activity in L6 myotubes by a mechanism independent of PI3K and Akt1/PKBα. The mechanism of action of these agents may involve a separate signaling pathway which converges with that of insulin.
CHAPTER TWO

Role of Akt1/PKBα in Vanadate and Pervanadate Stimulated Glucose Uptake

2.1 Introduction

Vanadate (VO₄³⁻) is an oxidized form of vanadium (45) and structurally resembles phosphate (46). Vanadate is a protein tyrosine phosphatase (PTP) inhibitor (47) and has been shown to mimic the action of insulin on glucose uptake and metabolism in insulin target tissues in vitro (48). In vivo, vanadate has been used as a therapeutic agent in several rodent models of Type I and Type II diabetes, and improved glucose control in human subjects with Type I and Type II diabetes (49). Upon mixing vanadate with hydrogen peroxide (H₂O₂), a peroxovanadium compound is formed termed pervanadate (pV) (50). pV is a potent PTP inhibitor, and like vanadate, has been shown to mimic the metabolic actions of insulin both in vitro and in vivo (51,52). Tsiani et al. (53) have shown that vanadate and pV stimulate glucose transport in cultured L6 myotubes in a time and dose dependent manner.

The mechanism of action of vanadate and pV remains uncertain. The effects of vanadate or pV on glucose transport in L6 myotubes are not additive with insulin suggesting that these agents activate the same signaling pathway or different pathways that converge (53). Treatment of cells with pV stimulates the insulin receptor tyrosine kinase and increases the overall cellular tyrosine phosphorylation of proteins, including IRS-1 (54), most likely due to powerful inhibition of PTPs (51). Vanadate and pV have been shown to activate PI3K (43). Previous work in our laboratory has shown that vanadate and pV stimulate glucose uptake in L6 myotubes to the same extent as insulin
but independently of PI3K (43). In this study (43), L6 myotubes were pretreated with or without the PI3K inhibitor wortmannin prior to stimulation by insulin, vanadate, or pV. Wortmannin is a fungal metabolite that binds irreversibly to the p110 catalytic subunit of PI3K (55) thereby abolishing the ability of the enzyme to produce 3-phosphoinositide lipid products. In the absence of wortmannin, all 3 agents activated PI3K and stimulated glucose transporter translocation and glucose uptake. Wortmannin pretreatment inhibited subsequent PI3K activation by all three agents but only the insulin-stimulated glucose uptake was inhibited. Vanadate and pV mediated glucose uptake was unaffected by wortmannin pretreatment. Therefore PI3K activation is important for insulin, but not vanadate or pV mediated glucose transport.

In addition to PI3K, Akt/PKB can also be activated by stimuli which do not activate PI3K. Okadaic acid, a serine/threonine phosphatase inhibitor, increases Akt/PKB activity and glucose transport in 3T3-L1 adipocytes and isolated rat adipocytes, but does not activate PI3K (39, 56). Cellular stress induced by heat shock and hyperosmolarity (57), agents which raise intracellular cAMP levels (58), and isoproterenol (59), have all shown to increase Akt/PKB activity in the presence of the PI3K inhibitor wortmannin. These findings suggest an alternate pathway(s) leading to activation of the kinase.

Since Akt/PKB has been implicated in the stimulation of Glut4 translocation by insulin, and various agents can activate the kinase independently of PI3K, the purpose of the following study was to determine whether the insulin mimetic agents vanadate and pV activated Akt/PKB independently of PI3K in L6 myotubes. The rationale for this study was based on previous findings in our laboratory which showed that vanadate and
pV stimulated glucose uptake and glucose transporter translocation in L6 myotubes by a mechanism independent of PI3K (43).

2.2 Materials and Methods

2.2.1 Materials

α-Minimum essential medium (α-MEM), fetal bovine serum (FBS), and antibiotics were obtained from Gibco (Burlington, ON). Sodium orthovanadate and wortmannin were purchased from Sigma (St. Louis, MO). Human insulin was a gift from Eli Lilly (Indianapolis, IN). Antibodies against the PH domain of Akt1/PKBα, and full length PKB/Akt were obtained from Upstate Biotechnology (Lake Placid, NY) and Dr. J. Woodgett (Ontario Cancer Institute, Toronto, ON) respectively. The antibody recognizing Serine 473 phosphorylated Akt1/PKBα was purchased from New England Biolabs (Boston, MA). 32P-ATP(3000Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA). The Akt1/PKBα Immunoprecipitation and Kinase Assay Kit was purchased from Upstate Biotechnology. Anti-rabbit secondary antibodies conjugated to horseradish peroxidase, and the enhanced chemiluminescence (ECL) reagent were purchased from Amersham (Oakville, ON). Nitrocellulose membranes (0.45μm) were obtained from Schleicher & Schuell (Keene, NH).

2.2.2 Cell Culture

L6 myoblasts were grown in 175cm² flasks in α-MEM containing 10% (vol/vol) FBS and 1% (vol/vol) antibiotic-antimycotic (10,000 U/ml penicillin G, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin B). Upon reaching confluence the cells were
trypsinized (0.25% trypsin) and transferred to 15cm petri dishes where they were grown in 2% (vol/vol) FBS and 1% (vol/vol) antibiotic-antimycotic. The cells were harvested once they had differentiated into myotubes.

2.2.3 Drug Treatments

A 25mM stock solution of vanadate was prepared in a 50mM HEPES buffer. pV was prepared by mixing 100μM of vanadate from the stock with 100μM H2O2. Prior to the drug treatments, the cells were washed twice with HEPES-buffered saline (HBS) (140mM NaCl, 5mM KCl, 20mM HEPES, 2.5mM MgSO4, 1mM CaCl2, pH 7.4) and preincubated in the presence or absence of 100nM wortmannin for 10 minutes. This concentration of wortmannin has been shown to effectively inhibit PI3K activity and the production of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 (43). Following the preincubation, the cells were stimulated with either 100nM insulin for 5 minutes, 5mM vanadate for 30 or 60 minutes, or 100μM pV for 15 minutes. All incubations were done at 37°C in HBS containing 5mM glucose. The control sample was incubated for 25 minutes. Following the treatments, the cells were washed two times with ice-cold HBS, scraped, and lysed with Buffer A (50mM Tris-HCl pH 7.5, 1mM EDTA, 1mM EGTA, 0.5mM Na3VO4, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50mM NaF, 5mM Na-pyrophosphate, 10mM sodium β-glycerophosphate, 0.1mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, pepstatin, leupeptin, and 1μM microcystin). The lysates were centrifuged 10,000 x g for 10 minutes. The supernatant was extracted, snap frozen in liquid nitrogen, and stored at -80°C until further analysis.

2.2.4 Akt1/PKBα Immunoprecipitation and Kinase Assay
Akt/PKB immunoprecipitation and kinase activity was determined from cell lysates. Briefly, Akt1/PKBα was immunoprecipitated from cell lysates by incubating 200μg of cell lysate with 4μg of antibody against the PH domain of Akt1/PKBα bound to Protein G agarose for 90 minutes. Following the incubation, the samples were centrifuged for 3 minutes at 10,000 X g. The immunocomplexes were then washed 3X with 500μl of Buffer A (Section 2.2.3) containing 0.5M NaCl, 2X with 500μl of Buffer B (50mM Tris-HCl, pH 7.5, 0.03% (w/v) Brij-35, 0.1mM EGTA and 0.1% 2-mercaptoethanol), and 1X with 100μl of Assay Dilution Buffer (20mM MOPS, pH 7.2, 25mM β-glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol). To determine kinase activity, the immunoprecipitates were incubated in a shaking water-bath for 10 minutes at 30°C with [γ32P] ATP (10μCi), 20mM MOPS, pH 7.2, 25mM β-glycerol phosphate, 5mM EGTA, 5mM sodium orthovanadate, 5mM dithiothreitol, cAMP-dependent protein kinase inhibitor peptide (10μM), and the substrate peptide (RPRAATF) (100μM), which is a relatively specific substrate for Akt1/PKBα because it is not phosphorylated to a significant extent by MAPKAP-K1 or p70 S6 kinase (60). Following the 10 minute incubation, the samples were centrifuged and the supernatants were spotted on P81 phosphocellulose paper. Radioactivity was determined by scintillation counting.

2.2.5 Immunoblot Analysis

Cells were serum starved for 3 hours prior to stimulation. Cell lysates (60μg) were subjected to SDS-PAGE (10% polyacrylamide) followed by electro transfer of proteins to nitrocellulose. Following the transfer, the membranes were incubated for 1 hour at 22°C.
in Tris buffered saline (TBS) (25mM Tris, pH 7.5, 154mM NaCl) containing 0.1% Tween 20 and 5% non-fat dry milk. The membrane was then incubated overnight with a polyclonal phospho-specific anti-S473 antibody to detect active Akt1/PKBα, or with an antibody against the full length of the kinase to quantify total levels. Following the overnight incubation the membrane was treated for 1 hour with an anti-rabbit secondary antibody conjugated to horseradish peroxidase. Proteins were then visualized by enhanced chemiluminescence and quantified by NIH Image.

2.2.6 Statistical Analysis

Results are expressed as MEANS ± SE. The significance between groups was determined using analysis of variance (ANOVA, p<0.05).

2.3 Results

Stimulation with 100nM insulin for 5 minutes resulted in a 2.30±0.37 fold activation of Akt1/PKBα above basal (Fig. 2.1). Treatment of cells with 5mM vanadate for 30 minutes resulted in a slight but insignificant activation of Akt1/PKBα, 1.15±0.17 above control, while stimulation with 5mM vanadate for 60 minutes did not activate Akt1/PKBα, 0.90±0.10. pV was a potent stimulator of Akt1/PKBα, 17±2.11 fold activation was observed after incubating the cells with 100μM pV for 15 minutes.
FIG. 2.1 Effect of insulin, vanadate, and pV on Akt1/PKBα activity. L6 myotubes were incubated in the presence or absence of 100nM wortmannin for 10 minutes. The cells were then stimulated with either 100nM insulin for 5 minutes, 5mM vanadate for 30 or 60 minutes, or 100μM pV for 15 minutes. Cells were lysed and Akt1/PKBα activity was determined using the immunoprecipitation kinase assay described in Section 2.2.4. Insulin and pV increased Akt1/PKBα activity which was inhibited by wortmannin pretreatment. Vanadate had no significant effect. Results are MEAN±SE of three to five experiments (*p < 0.05 compared to untreated Control).
Pretreatment with 100nM wortmannin for 10 minutes blocked Akt1/PKBα activation by insulin (1.08±0.26), vanadate (30 minutes, 0.86±0.11), and pV (1.80±0.57). Although not statistically significant, pretreatment of control cells with wortmannin reduced Akt1/PKBα activity in the basal state (0.88±0.19). Similar results were obtained by immunoblotting with an anti-phospho-Ser473 antibody which recognized the phosphorylated form of Akt1/PKBα (Fig.2.2).

2.4 Discussion

Insulin stimulates Akt1/PKBα activity in a time and dose dependent manner (61). The stimulation protocol used in this study (100nM insulin for 5 minutes) would produce maximal activation of the kinase by the hormone. Pretreatment of cells with wortmannin inhibited subsequent activation of the kinase by insulin. This finding is well documented and supports Akt1/PKBα as a downstream target of PI3K. Kohn et al. (38) have shown that inhibition of insulin stimulated Akt1/PKBα activity by using several concentrations of wortmannin closely paralleled the inhibition of PI3K in anti-phosphotyrosine immunoprecipitates, indicating that Akt1/PKBα activation by insulin is closely associated with the activation of PI3K.

pV was a potent activator of Akt1/PKBα and induces phosphorylation of Serine473. This observation is consistent with other reports (62,63). pV has been shown to potently activate and phosphorylate Akt1/PKBα by a wortmannin sensitive mechanism.
FIG. 2.2 L6 myotubes were pretreated in the presence or absence of 100nM wortmannin followed by the addition of either 100nM insulin for 5 minutes, 5mM vanadate for 30 minutes, or 100μM pV for 15 minutes. Cells were then lysed and proteins (60μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with (A) an anti-Ser473 antibody to examine phosphorylated Akt1/PKBα, or (B) an anti-Akt1/PKBα antibody to determine total levels. Insulin and pV increased the Ser473 phosphorylation of Akt1/PKBα, which was prevented by wortmannin. Vanadate treatment had a minimal effect. Total levels of Akt1/PKBα were similar in all conditions. Relative intensities of stimulation corrected for total Akt1/PKBα determined by densitometry in arbitrary units; insulin, 0.64; vanadate, 0.065; pV, 1.32.
in both 3T3 fibroblasts (62) and isolated rat adipocytes (63). In isolated rat adipocytes (63) pV stimulated Akt1/PKBα activity more potently than vanadate. Taken together, the results of the present study and previous studies indicate that pV, and to a much lesser extent vanadate, induce phosphorylation and activation of Akt1/PKBα by a PI3K mechanism.

The difference in activation of Akt1/PKBα by vanadate and pV may be due to the different mechanism of PTP inhibition by each agent. pV is a much more potent PTP inhibitor than vanadate (51,52). Vanadate, a completely reversible inhibitor, inhibits PTPs by competing with phosphate for binding at the active site (64). In contrast, pV inhibits PTPs irreversibly (64) by oxidizing the cysteine residue located at the catalytic site (65). The irreversible inhibition by pV may account for the fact that pV is much more potent in mimicking the actions of insulin than vanadate. Fantus et al. (52) have shown that pV is $10^3$-$10^3$ times more potent than vanadate in stimulating lipogenesis, protein synthesis, and inhibiting epinephrine-stimulated lipolysis in isolated rat adipocytes. In isolated rat skeletal muscle, pV treatment stimulated glucose utilization significantly more than vanadate alone (66). In addition, due to the different mechanisms of inhibition, vanadate and pV may have different PTP specificities. Fantus et al. (52) have shown that vanadate, but not pV, inhibited tyrosine dephosphorylation of the insulin receptor β-subunit by alkaline phosphatase. In the same experiment, pV powerfully inhibited tyrosine dephosphorylation of the insulin receptor by a crude preparation of phosphotyrosine phosphatase, while vanadate had minimal effects. In the case of the present study, pV may be inhibiting a key phosphatase(s) important in regulating the activation of Akt1/PKBα.
Pretreatment of L6 myotubes with wortmannin inhibited insulin stimulated Glut translocation and glucose uptake (43). Pretreatment with wortmannin at the concentration and duration used in this study effectively inhibited PI3K but did not block vanadate and pV induced glucose transport or Glut4 translocation (43), suggesting that Akt1/PKBα is also not essential for these agents to increase glucose transport.

It is interesting to note that other stimuli can also increase cellular glucose uptake by a pathway(s) that does not involve PI3K or Akt1/PKBα. Examples are muscle contraction (67,68), hypoxia (67) and osmotic shock (69). Muscle contraction and hypoxia have been shown to stimulate Glut4 translocation and glucose uptake as potently as insulin (67). Lee et al. (67) have shown that wortmannin pretreatment blocked insulin but not contraction or hypoxia stimulated glucose uptake in rat skeletal muscle. Furthermore, Brozinick et al. (68) have shown that muscle contraction does not activate Akt1/PKBα suggesting that contraction induced increases in glucose transport are not mediated by Akt/PKB. Chen et al. (69) have shown that osmotic shock increases glucose transport and Glut translocation in 3T3-L1 adipocytes by a wortmannin insensitive tyrosine kinase pathway that did not involve Akt/PKB.

In summary, pV, but not vanadate, potently activates Akt1/PKBα in L6 myotubes by a wortmannin sensitive mechanism. In contrast to insulin, both vanadate and pV stimulate glucose transport and Glut translocation by a mechanism independent of PI3K and Akt1/PKBα in L6 myotubes. Since glucose transport is not further stimulated by these agents above that achieved by maximum concentration of insulin, this mechanism appears to involve a separate signaling pathway that converges with the insulin action.
pathway (53). This is supported by the finding that cytochalasin D, an actin cytoskeleton disrupting agent, inhibited glucose transport stimulation by all three agents (53).
CHAPTER THREE

The Antioxidant N-Acetyl-Cysteine Prevents the Impairment in Glucose Transporter Translocation and Akt1/PKBα Activation in Hyperglycemia and Glucosamine Induced Insulin Resistance
ABSTRACT

To examine the potential role of oxidative stress in high glucose/insulin and glucosamine induced insulin resistance, rat adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin (High G/I) or 5mM glucosamine (Glc). Each incubation was done in the presence or absence of 5mM N-acetyl-cysteine (NAC). High G/I and Glc impaired the translocation of Glut4 from low-density microsomes (LDM) to the plasma membrane (PM), which could be prevented by NAC. High G/I, but not Glc treatment, decreased Glut1 expression in the PM, which was also prevented by NAC. Further examination of the insulin signaling cascade in High G/I treated cells revealed that IRS-1 tyrosine phosphorylation and association with the p85 subunit of PI3K in response to insulin was not impaired, whether examined in the LDM or in total cell lysates. In contrast, Akt1/PKBα activation and Ser473 phosphorylation in response to insulin were significantly impaired in resistant cells, which was prevented upon co-incubation with NAC.

In conclusion, NAC, an antioxidant and glutathione (GSH) precursor was able to prevent both hyperglycemia and glucosamine induced defects in insulin action. The results from this study suggest a role for oxidative stress in the induction of insulin resistance in this model. NAC may serve as a potential design for novel therapeutic agents.
CHAPTER THREE

The Antioxidant N-Acetyl-Cysteine Prevents the Impairment in Glucose Transporter Translocation and Akt1/PKBα Activation in Hyperglycemia and Glucosamine Induced Insulin Resistance

3.1 Introduction

Diabetes mellitus is a metabolic disease affecting 5% of the population worldwide (2, 70). Type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) accounts for over 90% of diabetic patients (2,70) and is characterized by (1) insulin resistance in peripheral target tissues, namely, muscle and fat, (2) decreased pancreatic β cell function, and (3) increased hepatic glucose production (71). Chronic complications of diabetes include kidney failure, lower limb amputations, blindness, and increased risk of cardiovascular disease and stroke (2,70).

Genetic and environmental factors contribute to the development of Type 2 diabetes (70). One of the earliest defects observed, and likely a key factor in the progression of the disease, is insulin resistance (70). Insulin resistance can be genetic or acquired or both, and is characterized by impaired stimulation of glucose uptake by insulin in target tissues (71). The exact cause or mechanism of insulin resistance is not entirely known. Genetic analysis of insulin resistance examined the effects of mutations in the insulin receptor gene and genes encoding the proteins in the insulin action pathway (2). Mutations in the insulin receptor gene result in severe insulin resistance, but this defect accounts for only a very small percentage of Type II diabetic patients (2). Type II diabetes has not been shown to be associated with mutations in genes encoding proteins in the insulin action pathway (2). At the present time, the genetic cause of insulin
resistance is not known and multiple inherited traits may be important (2). Numerous factors have been shown to be able to induce insulin resistance in target tissues such as hormones, cytokines, and metabolites (72). One of them is hyperglycemia (73). In Type II diabetic patients, hyperglycemia is present, and may exacerbate the already present insulin resistance (71).

3.1.1 Pathogenesis of Hyperglycemia-Induced Insulin Resistance: The Hexosamine Biosynthesis Pathway

In the late 1980s, Garvey et al. (73) showed using primary cultured rat adipocytes that insulin and glucose co-regulate the glucose transport system. In these experiments, isolated adipocytes were incubated in media for 24 h in the absence or presence of various glucose and insulin concentrations. At the end of the incubation, basal and maximal insulin stimulated glucose transport was assayed. Glucose alone had no effect on basal or maximal insulin stimulated glucose transport. Incubation of adipocytes with increasing concentrations of insulin in the absence of glucose slightly decreased insulin stimulated glucose transport but only at the highest insulin concentration tested, with no decrease in basal uptake. In contrast, co-incubation with glucose and insulin impaired subsequent basal and insulin stimulated glucose uptake in a dose-dependent manner. At the highest glucose and insulin concentration tested (20mM glucose, 50ng/ml insulin), basal and insulin stimulated glucose transport were decreased by 89% and 63% respectively. In a subsequent study by Traxinger et al. (74), it was discovered that insulin resistance induced by glucose and insulin required the presence of the amino acid L-glutamine. Together these findings led to the hypothesis that the hexosamine
biosynthesis pathway (HBP) was important in the induction of insulin resistance by high glucose (75). The HBP is thought to act as a ‘glucose sensor’ coupled to a negative feedback system which would sense high glucose availability and subsequently prevent excess glucose from entering the cell (75).

When adipocytes are exposed to high concentrations of glucose and insulin, (insulin facilitates glucose entry into cells), the incoming glucose saturates the glucose utilizing pathways such as glycogen synthesis and glycolysis. The remaining glucose (usually 2-3% of total incoming glucose) fluxes through the HBP. Glucose enters the cell by the glucose transporters and is phosphorylated to glucose-6-phosphate which can enter the glycogen synthesis pathway or be further processed to fructose-6-phosphate. Fructose-6-phosphate then enters the glycolytic pathway or the HBP. The first step of the HBP is the conversion of fructose-6-phosphate in the presence of L-glutamine to glucosamine-6-phosphate by the rate-limiting enzyme Glutamine: fructose-6-phosphate amidotransferase (GFA). Glucosamine-6-phosphate then undergoes a series of biochemical steps to form UDP-N-acetyl-hexosamines as the end products. The main product of this pathway, UDP-N-acetyl-glucosamine, is used in the synthesis of glycoproteins, proteoglycans, and glycolipids (Fig. 3.1). Several lines of evidence support the role of the HBP in high glucose induced insulin resistance. First, GFA inhibitors, azaserine and 6-diazo-5-oxonorleucine, which inhibit the conversion of fructose-6-phosphate to glucosamine-6-phosphate, also prevented insulin resistance induced by high glucose (75). This inhibition of GFA by azaserine closely paralleled the prevention of glucose induced insulin resistance (75). Second, overexpression of GFA in
FIG. 3.1 THE HEXOSAMINE BIOSYNTHESIS PATHWAY
skeletal muscle and adipocytes in transgenic mice resulted in decreased insulin mediated glucose disposal in vivo in the absence of hyperglycemia (76). Third, adipocytes treated with glucosamine become insulin resistant. Glucosamine enters adipocytes via the glucose transporters. Once inside the cell, glucosamine is phosphorylated to glucosamine-6-phosphate and enters the HBP distal to GFA, thereby bypassing the enzyme (75). Glucosamine is 40x more potent than glucose in inducing insulin resistance (75). Lastly, hyperglycemia and glucosamine induced insulin resistance are non-additive suggesting that both agents operate through the same pathway (77).

In addition to primary cultured rat adipocytes, chronic hyperglycemia and glucosamine treatment have been shown to induce insulin resistance in 3T3-L1 adipocytes (78) and in skeletal muscle preparations in vitro (79,80). In vivo, studies using the euglycemic hyperinsulinemic clamp technique have shown that prolonged hyperglycemia (81) or glucosamine infusions (81,82) markedly impair insulin mediated glucose uptake at the whole body level and in skeletal muscle and adipose tissue (82).

3.1.2. Potential Defects in the Insulin Signaling Pathway

Insulin resistance induced by chronic hyperglycemia and glucosamine treatment in isolated rat adipocytes is associated with impaired translocation of Glut4 to the plasma membrane without a change in total transporter number (73). Impaired Glut4 translocation has also been demonstrated in skeletal muscle (83), and in GFA overexpressing transgenic mice (76). This type of defect is also seen in Type II diabetic patients. Using NMR spectroscopy, Cline et al. (84) have shown that glucose transport stimulation by insulin is defective in these patients, which was not due to decreased

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levels of Glut4 (85). These findings suggest that impaired translocation of Glut4 to the plasma membrane is due to defective insulin signaling (83).

Studies examining the more proximal steps in the insulin signaling cascade in hyperglycemia and glucosamine induced resistance have led to controversial results. Isolated rat adipocytes exposed to chronic hyperglycemia displayed decreased activation of the receptor tyrosine kinase and IRS-1 tyrosine phosphorylation in response to insulin in one study (86). Another study using the same cells and similar incubation conditions showed no defect in insulin receptor kinase activity or IRS-1 phosphorylation. Prolonged exposure of rat skeletal muscle to 25mM glucose in vitro impaired Akt/PKB activation and phosphorylation by insulin while PI3K activation was unaffected (79). Glucosamine treatment of 3T3-L1 adipocytes led to impaired insulin receptor tyrosine kinase activity and IRS-1 tyrosine phosphorylation, impaired IRS-1 associated PI3K activity and decreased Akt/PKB activation by insulin (87). In vivo, chronic glucosamine infusion in rats did not decrease insulin receptor tyrosine phosphorylation in skeletal muscle (88, 89). IRS-1 tyrosine phosphorylation in response to insulin was reduced in one study (90) but not in another (88). Glucosamine treatment has been shown to reduce IRS-1 associated PI3K activity while having no effect on Akt/PKB activation or phosphorylation (88,89). Similar findings were recently shown in skeletal muscle of obese Type II diabetic patients (90). Obese Type II diabetic subjects displayed normal Akt1/PKBα and Akt2/PKBβ activation and phosphorylation in response to insulin while IRS-1 and IRS-2 associated PI3K activities were significantly reduced compared to lean controls and obese non-diabetic subjects. The reason for the discrepancies among the various studies
and treatments is presently not clear but may be due to the length of the treatment times or the presence or absence of insulin during the treatment.

3.1.3 Mechanisms of Hexosamine Induced Insulin Resistance

How the increased flux through the HBP with the accumulation of UDP-N-acetylglucosamine impairs Glut4 translocation is not known but several potential mechanisms have been proposed. One mechanism is the activation of protein kinase C (PKC).

Indirect evidence stems from the finding that the decrease in insulin receptor tyrosine phosphorylation induced by high glucose in rat adipocytes was prevented by coinubcation with a PKC inhibitor (86). Hyperglycemia has been shown to activate PKC (86) likely by increasing intracellular diacylglycerol (DAG) levels (91). DAG can be produced from the glycolytic intermediates dihydroxyacetone phosphate and glycerol 3-phosphate (91). With increasing glucose concentrations, more glucose is available for glycolysis thereby increasing cellular DAG levels. Some evidence implicating PKC in hexosamine-induced resistance comes from a study done by Filippis et al. (92). In this study, exposure of rat adipocytes to high glucose or glucosamine activated PKC. Pretreating the cells with the GFA inhibitor azaserine almost completely prevented the activation of PKC by high glucose while having no effect on glucosamine induced PKC activation. The residual elevated PKC activity remaining in the presence of azaserine treatment may have been due to DAG production from glycolysis, suggesting that hexosamines are powerful PKC activators (92). In this same study, treatment of cells with the PKC inhibitor R0-31-8220 prevented insulin resistance induced by these agents.
It has been suggested that activation of PKC may lead to serine/threonine phosphorylation of the insulin receptor thereby decreasing its tyrosine kinase activity towards endogenous substrates such as the IRS proteins (93), and/or that the IRS proteins are themselves substrates for PKC which inhibit their tyrosine phosphorylation by the receptor (94).

Increased flux through the HBP increases the intracellular concentration of UDP-N-acetylglucosamine, which serves as a substrate for O-linked glycosylation of various proteins (72). Increased O-linked glycosylation may affect the activity of transporters and enzymes involved in insulin signaling (72). Haltiwanger et al. (95) have shown that increased O-linked glycosylation of the transcription factor Sp1 resulted in a reciprocal decrease in its level of phosphorylation suggesting that O-linked N-acetylglucosamine may compete with phosphate on some proteins. Recently, Patti et al. (89) have shown that glucosamine infusion resulted in O-linked glycosylation of IRS-1 and IRS-2 in rat skeletal muscle. UDP-N-acetylglucosamine may also modify gene transcription. Increasing intracellular concentrations of UDP-N-acetylglucosamine in adipocytes and skeletal muscle by high glucose or glucosamine treatment resulted in rapid and marked increases in leptin messenger RNA and protein levels (96). To date, the significance of enhanced O-linked protein glycosylation in the induction of insulin resistance remains uncertain.

3.1.4 Potential Role of Oxidative Stress in Insulin Resistance

Oxidative stress has been implicated in the development of insulin resistance and diabetic complications (97) and results from either an overproduction of reactive oxygen
free radicals and/or from a decreased efficiency of radical scavenger systems (97). The net result is an increase in intracellular oxygen free radicals, which can be formed in cells by many processes (98). Oxygen radicals are capable of reversibly or irreversibly damaging nucleic acids, proteins, and lipids, thereby disrupting normal cell function (98). Oxidative stress has been implicated in the development of numerous human diseases (98) including Type II diabetes. Evidence to support a role of oxidative stress in the development of insulin resistance and Type II diabetes comes from data from Type II diabetic subjects, and numerous in vitro and in vivo studies.

Oxidative damage to DNA and membrane lipids has been observed in Type II diabetic patients. Type II diabetic subjects displayed higher levels of 8-OHdG (8-hydroxydeoxyguanosine), an indicator of oxidative damage to DNA in the plasma and urine (99,100). These subjects also displayed higher levels of lipid peroxidation products in the plasma and urine, suggesting damage to membrane lipids (101). In addition, plasma free radical (O\textsuperscript{2-}) concentrations were negatively correlated with whole body glucose disposal (r = -0.53, ref. 97) in diabetic subjects, suggesting that free radicals were associated with insulin resistance.

Hyperglycemia and elevated free fatty acids (FFA), conditions present in Type II diabetes, are known to cause insulin resistance in target tissues and have also been shown to cause oxidative stress. Hyperglycemia is a well-known cause of enhanced plasma free radical concentrations (102). Cells exposed to prolonged hyperglycemia displayed oxidative damage to DNA and increased levels of lipid peroxidation products (100). Plasma free fatty acids have also been shown to correlate with plasma free radicals (103). In healthy human subjects, increasing the plasma free fatty acid concentration by a 24 h
infusion of intralipid caused an increase in malondialdehyde concentration (an indicator of oxidative stress), and a decline in plasma GSH/GSSG ratio (reduced form of glutathione/oxidized form of glutathione). Hyperlipidemia has been shown to cause insulin resistance in skeletal muscle, likely by interfering with the glucose/fatty acid cycle (81). Interestingly, intralipid infusions in rats led to an increase in skeletal muscle UDP-N-acetylglucosamine levels suggesting that free fatty acids may induce insulin resistance by increasing the flux through the HBP, similar to high glucose (81).

*In vitro*, induction of oxidative stress by prolonged exposure of 3T3-L1 adipocytes to micromolar concentrations of hydrogen peroxide (H$_2$O$_2$) impaired Glut4 translocation to the plasma membrane in response to insulin (104) and depleted the intracellular levels of reduced glutathione (GSH) (105). Treatment of 3T3-L1 adipocytes with lipoic acid, an antioxidant, prior to treatment with H$_2$O$_2$ prevented the impairment in Glut4 translocation and maintained GSH levels (105).

In light of the findings that hyperglycemia and hyperlipidemia are associated with oxidative stress (102,103) and increase cellular UDP-N-acetylhexosamines (81), and that H$_2$O$_2$ impaired Glut4 translocation in 3T3-L1 adipocytes (104), our laboratory has investigated whether insulin resistance induced by high glucose or glucosamine is due to oxidative stress. To explore this possibility, isolated rat adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin (High G/I), or 5mM glucosamine (Glc). The incubations were done in the presence and absence of 5mM N-acetyl-cysteine (NAC). NAC is an antioxidant and glutathione (GSH) precursor (44), which would scavenge any reactive oxygen species produced. High G/I decreased both basal
Basal Glc treatment did not significantly reduce basal glucose uptake (control 307±29; Glc 254±54), but the insulin stimulated glucose uptake was reduced (control 949±101, Glc 461±75) which was prevented by NAC (Glc + NAC basal 335±63; insulin-stimulated 1049±98, p<0.01).

The present study examined the effect of NAC on Glut4 translocation and Glut1 expression in adipocytes treated with High G/I and Glc. To gain further insight into the mechanism of NAC action in adipocytes treated with high G/I, insulin induced IRS-1 tyrosine phosphorylation, IRS-1 association with the p85 subunit of PI3K, and Akt1/PKBα activation were examined. These measurements were performed in order to determine which signaling event(s) were altered, and the effect of NAC on these steps in the insulin-signaling cascade.

3.2 Materials and Methods

3.2.1 Materials

Dulbecco’s Modified Eagles Medium (DMEM) was obtained from Gibco (Burlington, ON, Canada). Collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Bovine serum albumin (BSA), N-acetyl-cysteine (NAC), and the 5’-nucleotidase assay kit were obtained from Sigma (St. Louis, MO). Human insulin was a gift from Eli Lilly (Indianapolis, IN). Antibodies against Glut4 and Glut1 were a kind gift from Dr. Samuel Cushman (National Institutes of Health). Antibodies against IRS-1,
the p85 subunit of PI3K, and the Akt1/PKBα substrate Crosstide were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine antibodies (pY99) were obtained from Santa-Cruz Biotechnology. Protein A Sepharose was purchased from Pharmacia Biotechnology.

3.2.2 Adipocyte Isolation and Preparation

The method used to obtain isolated adipocytes was as described by Rodbell (106) with minor modifications (73). Sprague-Dawley rats weighing 200-250g were killed by CO₂ inhalation followed by cervical dislocation. The epididymal fat pads were removed and placed in DMEM containing 25mM HEPES, 1% antibiotic-antimycotic solution, 0.5% FBS, and 3% BSA. Isolated adipocytes were obtained by adding collagenase (4mg/ml) to the medium and shaking for 1 h at 37°C. The cells were then filtered through nylon and washed 2 times with the same medium described above followed by 2 more washes with DMEM containing 25mM HEPES, 1% antibiotic solution, 0.5% FBS, and 1% BSA.

3.2.3 Primary Culture of Adipocytes

Stock solutions of glucosamine and NAC were prepared fresh before each experiment and equilibrated at pH 7.5. Freshly isolated adipocytes were incubated for 18 h at 37°C in DMEM containing 25mM HEPES, 1% antibiotic solution, 0.5% FBS, and 1% BSA. Cells were incubated either in media alone (5.6mM glucose), or containing 20mM D-glucose/100nM insulin (High G/I), High G/I plus 5mM NAC, 5mM glucosamine, or 5mM glucosamine plus 5mM NAC. Insulin, 4.2nM, was added to the glucosamine conditions to facilitate glucosamine entry into the cells. All incubations were done in
sterile polypropylene flasks and maintained in a humidified atmosphere of 95% air/5%CO₂ at 37°C. Following the 18 h incubation, the cells were washed twice with KR3OH (137mM NaCl, 5mM KCl, 1.2mM KH₂PO₄, MgSO₄, 1.25mM CaCl₂, 30mM HEPES, 1mM sodium-pyruvate, pH 7.0) containing 3% BSA, and then incubated in the same buffer for 30 minutes at 37°C to remove all extracellular and receptor bound insulin. This step allows the glucose transport system in the insulin treated cells to return to basal levels (73). Following the 30 minute incubation, the cells were washed twice in KRBH (118mM NaCl, 5mM KCl, 1.2mM MgSO₄, 2.5mM CaCl₂, 1.2mM KH₂PO₄, 5mM NaHCO₃, 30mM HEPES, 1mM sodium-pyruvate, pH 7.5) containing 3% BSA.

3.2.4 Subcellular Fractionation

To obtain plasma membranes (PM) and low-density microsomes (LDM), the cells were fractionated following a procedure modified from Simpson et al. (107). Following incubation with or without insulin at 37°C, the cells were washed once in TES buffer (25mM Tris pH 7.4, 2mM EDTA, 0.25M sucrose, 0.01 mg/ml aprotinin, 0.01mg/ml leupeptin, 0.1mm PMSF) previously equilibrated at 18°C, and homogenized rapidly using a glass-teflon homogenizer. All subsequent steps were performed at 4°C. Whole cell homogenates were centrifuged at 17,000 x g for 15 minutes. The fat cake was carefully removed and discarded. The supernatant (Supernatant 1) was removed and transferred to another tube. The pellet (Pellet 1) was re-suspendend in TES buffer and centrifuged again at 17,000 x g for 15 minutes. Supernatant 1 was centrifuged at 48,000 x g for 15 minutes. The pellet, containing the high-density microsomes, was discarded. The supernatant (Supernatant 2) was re-suspended in TES buffer and centrifuged at
400,000 x g for 60 minutes. The pellet obtained from this step contained the LDM. To obtain the PM fraction, Pellet 1 (the first pellet obtained) was resuspended in TES buffer and layered on a 1.12M sucrose cushion (1.12M sucrose, 2mM EDTA, 25mM Tris, pH 7.4) and centrifuged at 95,000 x g for 65 minutes in a swinging bucket rotor (SW60, Beckman). The PM fraction was collected at the interface between the two liquid layers, re-suspended in TES buffer, and centrifuged at 48,000 x g for 15 minutes. The pellet, containing the PM fraction was re-suspended in TES buffer and centrifuged again at the same speed. PM and LDM fractions were solubilized in Buffer A (Section 2.2.3) and stored at -80°C until further analysis. Total protein yields (Section 3.2.6) of LDM and PM fractions were similar in all conditions. The purity of the PM fraction was determined by 5'-nucleotidase activity as described in Section 3.2.11.

3.2.5 Preparation of Whole Cell Lysates

Following treatment with or without insulin, the cells were washed once at room temperature with KRBH (no BSA) and rapidly homogenized in Buffer A (Section 2.2.3) using a glass-teflon homogenizer. All subsequent steps were performed at 4°C. The homogenate was centrifuged at 10,000 x g for 10 minutes. The fat cake was carefully removed and discarded. The supernatant was transferred to another tube and stored at -80°C until further analysis.

3.2.6 Protein Determination

Protein levels were determined by the Bio-Rad method.
3.2.7 Determination of Glut4 Translocation and Glut1 Levels

Following the 18 h incubation and washing procedures, the cells were incubated in KRBH containing 3% BSA for 30 minutes at 37°C in the presence or absence of 100nM insulin. Following insulin treatment, LDM and PM fractions were obtained. LDM and PM samples (30µg) were separated by SDS-PAGE using 10% polyacrylamide, and transferred to nitrocellulose membranes. The membranes were first incubated at room temperature for 1 h in TBS (Section 2.2.5) containing 0.1% Tween-20, 5% non-fat dry milk to minimize non-specific binding, and then incubated for 16 h at 4°C in TBS containing 3% BSA with antibodies recognizing either Glut4 (1:1000) or Glut1 (1:2000). The membranes were then incubated for 1 h at room temperature in TBS (3% BSA) containing an anti-rabbit secondary antibody conjugated to horseradish peroxidase. Proteins were visualized by enhanced chemiluminescence. The intensities of the bands were quantified using NIH Image.

3.2.8 IRS-1 Immunoprecipitation

Cells were incubated for 5 minutes at 37°C in KRBH containing 3% BSA in the presence or absence of 100nM insulin. Following insulin stimulation, the cells were either fractionated to obtain the LDM or whole cell lysates were prepared. One hundred µl of washed Protein A sepharose bead slurry (50µl packed beads) was incubated with 4µg of α-IRS-1 for 2 h at 4°C in phosphate buffered saline (PBS) (137mM NaCl, 3mM KCl, 8mM Na₂HPO₄, 15mM KH₂PO₄, pH 7.4). LDM samples (500µg) or whole cell lysates (1mg) were added to the tubes which were then left shaking overnight at 4°C. Following the overnight incubation, the precipitated immunocomplex was washed 3 times with
500μl of ice cold PBS. The sepharose beads were re-suspended in 2x Laemmli sample buffer (108) and boiled for 5 minutes. The beads were then centrifuged at 10,000 x g for 1 minute. The supernatant was collected and analyzed.

3.2.9 Determination of IRS-1 Tyrosine Phosphorylation and Association with p85

The IRS-1 immunoprecipitated from the above step was divided into 2 aliquots. One aliquot was for the determination of tyrosine phosphorylation, and the other for association with p85. The aliquots were separated by SDS-PAGE by the procedure outlined in Section 3.2.7. To determine IRS-1 tyrosine phosphorylation, the membrane was first incubated at room temperature for 1h in TBS containing 0.1% Tween-20 with 5% BSA. Anti-phosphotyrosine antibodies (1:1000) were then added to the medium and the membrane was left shaking overnight at 4°C. The membrane was then incubated for 1 h at room temperature in TBS (3% BSA) containing an anti-mouse antibody conjugated to horseradish peroxidase. Total p85 associated with IRS-1 was determined by the procedure outlined in Section 3.2.7 with α-p85 (1:1000) as the primary antibody. To ensure that equal amounts of IRS-1 were immunoprecipitated in all conditions, the membranes were treated with a 15% H₂O₂ solution for 15 minutes at room temperature with continuous shaking. The membranes were then probed for total IRS-1 as described above. Since the amount of IRS-1 immunoprecipitated from the LDM was similar in all conditions, the densitometry values from the anti-phosphotyrosine immunoblots were used in the calculations. Due to slight variability in the amount of IRS-1 immunoprecipitated from total cell lysates, the anti-phosphotyrosine values were corrected for total IRS-1 present.
3.2.10 Akt1/PKBα Activation and Phosphorylation

Cells were incubated in KRBH (1% BSA) for 10 minutes at 37°C either in the presence or absence of 100nM insulin. Whole cell lysates were then prepared as described in Section 3.2.5. Akt1/PKBα activity was determined by the kinase assay procedure outlined in Section 2.2.4. The substrate Crosstide (30μM per assay) was used. Serine 473 phosphorylation of Akt1/PKBα was determined by western immunoblotting according to the procedure outlined in 2.2.5.

3.2.11 5'-Nucleotidase

5'-Nucleotidase (5'-ND) activity was determined by a spectrophotometric assay which measured the rate of nicotinamide adenine dinucleotide (NAD) formation from the following series of reactions (abbreviations defined below):

\[
\begin{align*}
5'\text{-ND} \\
\text{AMP} & \xrightarrow{\text{ADA}} \text{Adenosine + Pi} \\
\text{Adenosine} & \xrightarrow{\text{ADA}} \text{Inosine + Ammonia} \\
\text{Ammonia} + \text{2-Oxoglutarate} + \text{NADH} & \xrightarrow{\text{GLDH}} \text{L-Glutamate + NAD}
\end{align*}
\]

Samples (100μl) of whole cell lysates, LDM and PM fractions were added to 900μl of 5'-ND reagent containing 3.2mM adenosine monophosphate (AMP), 0.2mM reduced NAD (NADH), 3.7mM 2-Oxoglutarate, 11 000U/L L-glutamate dehydrogenase (GLDH), adenosine deaminase (ADA) 400 U/L, β-glycerophosphate, buffer, and stabilizers. Once added, the reaction mixture was mixed by inversion and left at room temperature for 5
After 5 minutes the absorbance (A) was read using a spectrophotometer (Beckman DU40) at 340nm versus water as a reference (Initial A). The mixtures were then placed in a 37°C incubator for 5 minutes after which the absorbance was read again (Final A). 5’ND activity was calculated as follows:

\[ \text{★A per 5 min (U/L)} = \text{Initial A} - \text{Final A} \]

\[ 5'\text{-ND} = \text{★A per 5 min X 322 X 1.35} \]

Where: Factor 322 = \( \frac{1 \times 1000}{6.22 \times 0.1 \times 5} \)

1 = Total reaction volume (ml)
1000 = Conversion of activity per ml to activity per L
6.22 = Millimolar absorptivity of NADH at 340nm
0.1 = Volume of sample (ml)
5 = Conversion of ★A per 5 min to ★A per min
1.35 = Temperature correction factor

Enzyme activities were then corrected for total protein in the reaction mixtures and expressed as U/L/mg protein. Results are: Whole cell lysate 10, LDM 6, PM 81.

3.2.12 Statistical Analysis

Results represent the MEAN±SE. To analyze the data, a one-way analysis of variance (ANOVA) was used. Post hoc comparisons of means were performed using Scheffe’s test and Tukey’s test. Statistical significance was defined as \( p < 0.05 \).
3.2 Results

3.3.1 Effect of NAC on Glut4 Translocation in Cells Treated with High G/I and Glucosamine

Glut4 levels in each fraction are expressed as a percentage of the total Glut4 found in the LDM and PM fractions combined, designated in each experiment as 100%. In basal cells, almost all of the Glut4 was found in the LDM with very little found in the PM. This was seen in all conditions (Fig. 3.2 and Fig. 3.3). In control cells, insulin treatment resulted in the translocation of Glut4 from the LDM to the PM, increasing the plasma membrane concentration of Glut4 with a corresponding decrease in the LDM content (Fig. 3.2 and Fig. 3.3). In cells treated with High G/I, Glut4 translocation to the PM was impaired (Insulin stimulated PM; Control 74.1±6.3%, High G/I 35.7±5.5%, \( p<0.05 \), \( n=3 \)) with Glut4 being retained in the LDM (Insulin stimulated LDM; Control 25.9±6.3%, High G/I 64.3±5.5%, \( p<0.05 \)). Co-incubation with NAC prevented the impairment in Glut4 translocation induced by High G/I (Insulin stimulated; PM 69.4±2.1%, LDM 30.6±2.1%). In cells treated with High G/I + NAC, the distribution of Glut4 between the LDM and PM compartments in response to insulin was similar to the control (Fig. 3.2). Similar results were seen in cells treated with glucosamine (Fig. 3.3). Glucosamine treatment impaired the translocation of Glut4 to the PM in response to insulin (Insulin stimulated PM; Control 71.3±4.4%, Glc 46.8±6.6%, \( p<0.05 \), \( n=3 \)) with Glut4 remaining in the LDM (Insulin stimulated LDM; Control 28.7±4.4%, Glc 53.2±6.6%, \( p<0.05 \)). Co-incubation with NAC prevented the impairment in Glut4 translocation induced by glucosamine treatment (Insulin stimulated; PM 79.3±6.01%, LDM 20.73±6.01%). Incubation with NAC alone had no effect on Glut4 translocation in
FIG. 3.2 Glut4 translocation in High G/I and NAC treated rat adipocytes. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC respectively). Cells were washed, and incubated for 30 min in the presence or absence of 100nM insulin. LDM and PM samples (30μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-Glut4 antibody. (A) Representative immunoblot. (B) Data are MEAN±SE of 3 independent experiments ( *p < 0.05, High G/I vs others).
FIG. 3.3 Glut4 translocation in Glc and NAC treated rat adipocytes. Adipocytes were incubated for 18 h in media containing 5mM glucosamine/4.2nM insulin in the absence and presence of 5mM NAC (Glc and Glc + NAC respectively). Cells were washed, and incubated for 30 min in the presence or absence of 100nM insulin. LDM and PM samples (30μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-Glut4 antibody. (A) Representative immunoblot. (B) Data are MEAN±SE of 3 independent experiments (*p < 0.05, Glc vs others).
response to insulin (Basal Control; LDM 85.0%, PM 15.0%, NAC; LDM 88.0%, PM 12.0%. Insulin Control; LDM 29.0%, PM 71.0%, NAC; LDM 28.0%, PM 72.0%) (Fig. 3.4).

3.3.2 Effect of NAC on Glut1 Expression in Cells Treated with High G/I and Glucosamine

Glut1 was mainly detected in the PM (Fig. 3.5 and Fig. 3.6), consistent with previous reports (27). Since Glut1 translocation to the PM in response to insulin was not observed, only basal cells were used in the calculations. All Glut1 data is expressed as fold of control basal which was designated a value of 1.0. In cells treated with High G/I, Glut1 levels were significantly reduced (Control 1.0, High G/I 0.2±0.1, p<0.05, n=3) (Fig. 3.5). This finding was consistent with the decrease in basal glucose uptake seen in cells treated with High G/I. Co-incubation with NAC prevented the downregulation in Glut1 induced by High G/I (High G/I + NAC 1.2±0.1) (Fig. 3.5). In contrast to High G/I, 18 h glucosamine treatment did not significantly reduce Glut1 levels and co-incubation with NAC had no further effect (Control 1.0, Glc 1.1±0.2, Glc + NAC 1.0±0.1, p = NS, n=3) (Fig. 3.6). Glucosamine treatment did not significantly affect basal glucose uptake. Treatment with NAC alone had no effect on Glut1 levels (Control 1.0, NAC 1.08)(Fig. 3.4).

3.3.3 Effect of High G/I and NAC on Akt1/PKBα Activation and Phosphorylation

Treatment of control cells with 100nM insulin for 10 minutes resulted in a 14±2.8 fold increase above basal in Akt1/PKBα activation (Fig. 3.7). In cells treated with High G/I, Akt1/PKBα activation by insulin was reduced by 44% compared to the insulin
FIG. 3.4 Effect of NAC alone on Glut4 translocation and Glut1 expression. Adipocytes were incubated for 18 h in media containing 5mM NAC. Cells were washed, and incubated for 30 minutes in the presence or absence of 100nM insulin. LDM and PM samples (30μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using (A) an anti-Glut4 antibody, or (B) an anti-Glut1 antibody.
FIG. 3.5 Glut content of plasma membranes in High G/I and NAC treated rat adipocytes. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC respectively). Cells were washed, and incubated for 30 min in the presence or absence of 100nM insulin. LDM and PM samples (30µg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-Glut1 antibody. (A) Representative immunoblot. (B) Data are MEAN±SE of 3 independent experiments (*p < 0.05, High G/I vs others). Results are expressed as the fraction of total Glut1 distributed in the PM in each condition, PM of Control was given the value 1.0.
FIG. 3.6 Glut1 content of plasma membranes in Glc and NAC treated rat adipocytes. Adipocytes were incubated for 18 h in media containing 5mM glucosamine/4.2nM insulin in the absence and presence of 5mM NAC (Glc and Glc + NAC respectively). Cells were washed, and incubated for 30 min in the presence or absence of 100nM insulin. LDM and PM samples (30µg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using an anti-Glut1 antibody. (A) Representative immunoblot. (B) Data are MEAN±SE of 3 independent experiments. Results are expressed as the fraction of total Glut1 distributed in the PM in each condition. Control PM was assigned the value 1.0.
FIG. 3.7 Effect of High G/I and NAC on Akt1/PKBα activity. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC respectively). Following the incubation, cells were washed, and incubated for 10 min in the presence or absence of 100nM insulin. Cells were then lysed, Akt1PKBα immunoprecipitated, and an in vitro kinase assay was performed using the substrate Crosstide. Results represent MEAN±SE of 4 independent experiments (*p<0.05, High G/I vs others).
stimulated control (High G/I; 7.8±0.95, p<0.05, n=4). Co-incubation with NAC prevented the decrease in Akt1/PKBα activation induced by High G/I (High G/I + NAC: 14.7±3.1) (Fig. 3.7). Basal Akt1/PKBα activity was not different among conditions. Incubation with NAC alone reduced insulin stimulated Akt1/PKBα activation by 34%, while having no effect on basal Akt1/PKBα activity (Basal: Control 0.09±0.03, NAC 0.05±0.01, p = NS, n=4. Insulin stimulated: control 1.0, NAC 0.66±0.05, p < 0.05, n=4) (Fig.3.8).

Immunoblot analysis of Ser473 phosphorylated Akt1/PKBα showed similar results (Fig. 3.9). In basal cells, Ser473 phosphorylated Akt1/PKBα was not detected, but upon stimulation with insulin a prominent band appeared on the immunoblot. Therefore, only insulin treated conditions were used in the calculations. The insulin stimulated control was designated a value of 1.0. In cells treated with High G/I, Akt1/PKBα phosphorylation on Ser473 was significantly decreased compared to the control (Control: 1.0, High G/I; 0.31±0.15, p<0.05, n=3) (Fig. 3.9). Co-incubation with NAC prevented the impairment in Akt1/PKBα phosphorylation seen in cells treated with High G/I (High G/I + NAC 1.18±0.07) (Fig. 3.9). Incubation with NAC alone did not have any effect on the insulin-induced phosphorylation of Ser473 (Control 1.0, NAC 1.14±0.02, p = NS, n=3) (Fig. 3.10). Total levels of Akt1/PKBα were unchanged by the treatments (Basal: Control 1.0, High G/I 1.04 ± 0.23, High G/I + NAC 1.0 ± 0.08, p = NS. Insulin stimulated; Control 0.97 ±0.08, High G/I 0.82 ±0.05, High G/I +NAC 0.90 ±0.08, p = NS, n=3) (Fig. 3.10 and Fig. 3.11).
FIG. 3.8 Effect of NAC on Akt1/PKBα activity. Adipocytes were incubated for 18 h in media containing 5mM NAC. Following the incubation, cells were treated with 100nM insulin for 10 minutes. Cells were subsequently lysed, Akt1/PKBα immunoprecipitated and in vitro kinase activity determined using the substrate Crosstide. Results represent MEAN±SE of 4 independent experiments. The insulin treated control was assigned the value 1.0 (Basal; Control 0.09±0.03, NAC 0.05±0.01, p = NS. Insulin; Control 1.0, NAC 0.66±0.05, *p<0.05).
FIG. 3.9 Effect of High G/I and NAC on Ser473 phosphorylation of Akt1/PKBα. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC, respectively). Following the incubation, the cells were washed, stimulated for 10 minutes with 100nM insulin, and lysed. Samples (60μg) were separated on SDS-PAGE, transferred to nitrocellulose, and probed for (A) Ser473 phosphorylated Akt1/PKBα. (B) Represents the MEAN±SE of 3 independent experiments. The insulin treated control was designated the value 1.0 since Ser473 phospho-Akt1/PKBα was only detected with insulin treatment (Control 1.0; High G/I 0.31±0.15; High G/I + NAC 1.17±0.07, *p<0.05, Control vs others).
FIG. 3.10 Effect of NAC alone on Ser473 phosphorylation of Akt1/PKBα. Adipocytes were incubated for 18 h in media containing 5mM NAC. Following the incubation, the cells were washed, stimulated for 10 minutes with 100nM insulin, and lysed. Samples (60μg) were separated on SDS-PAGE, transferred to nitrocellulose, and probed for (A) total Akt1/PKBα, or (B) Ser473 phosphorylated Akt1/PKBα. (C) Represents the MEAN±SE of 3 independent experiments represented in (B), the insulin treated control was designated the value 1.0 since Ser473 phospho- Akt1/PKBα was only detected with insulin treatment (Control 1.0; NAC 1.1±0.02, p = NS).
FIG. 3.11 Effect of High G/I and NAC on total levels of Akt1/PKBα. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC, respectively). Following the incubation, the cells were washed, stimulated for 10 minutes with 100nM insulin, and lysed. Samples (60µg) were separated on SDS-PAGE, transferred to nitrocellulose, and probed for (A) total Akt1/PKBα. (B) Represents the MEAN±SE of 3 independent experiments (Basal; control 1.0; High G/I 1.04 ± 0.23; High G/I + NAC 1.0 ± 0.08 p = NS. Insulin stimulated; control 0.97 ±0.08; High G/I 0.82 ±0.05, High G/I +NAC 0.90 ±0.08, p = NS).
3.3.4 Effect of High G/I and NAC on IRS-1 Tyrosine Phosphorylation and Association with p85 in the LDM

Several studies have suggested that insulin resistance is associated with increased serine/threonine phosphorylation of IRS-1, which may prevent its interaction with, or render it an inhibitor rather than a substrate of the insulin receptor kinase (94). This would decrease insulin-stimulated tyrosine phosphorylation (93, 109). To determine whether the High G/I treatment caused such a defect, IRS-1 was immunoprecipitated from the LDM and tyrosine phosphorylation measured. In rat adipocytes, it has been demonstrated previously that the majority of IRS-1 is located in the LDM and this localization was unchanged after insulin stimulation (36). This was confirmed in this study. Total levels of IRS-1 in the LDM were unchanged by any of the treatments (Basal; Control 1.0, High G/I 0.75±0.05, High G/I + NAC 0.81±0.07, p = NS. Insulin; Control 0.97±0.11, High G/I 0.78±0.02, High G/I + NAC 0.99±0.13, p = NS, n=3) (Fig. 3.12). Insulin treatment of control cells led to a 13-fold increase in tyrosine phosphorylation compared to basal cells (Control; Basal 0.06±0.03, Insulin treated control was designated 1.0) (Fig. 3.13). Treatment of cells with High G/I for 18 h did not have an effect on insulin-stimulated tyrosine phosphorylation of IRS-1 compared to the control (Insulin; High G/I 1.03±0.35, p = NS, n=3). There was a slight increase in basal tyrosine phosphorylation of IRS-1 compared to the basal control but this was not statistically significant (Basal; High G/I 0.22±0.07, p = NS). In cells co-incubated with NAC and High G/I, tyrosine phosphorylation of IRS-1 in basal and insulin treated cells was similar to the control (High G/I + NAC; Basal 0.03±0.01, Insulin 0.89±0.32, p = NS) (Fig. 3.13).
FIG. 3.12 Effect of High G/I and NAC on IRS-1 levels in the LDM. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC, respectively). Cells were subsequently washed and stimulated with 100nM insulin for 5 minutes. LDM samples (80µg) were resolved by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-IRS-1 antibody. (A) Representative immunoblot. (B) Represents the MEAN±SE of 3 independent experiments. Basal control was assigned the value 1.0 (Basal; Control 1.0, High G/I 0.75±0.05, High G/I + NAC 0.81±0.07, p = NS. Insulin; Control 0.97±0.11, High G/I 0.78±0.02, High G/I + NAC 0.99±0.13, p = NS).
FIG. 3.13 Effect of High G/I and NAC on IRS-1 tyrosine phosphorylation in the LDM. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC, respectively). Following the incubation, cells were washed and treated with 100nM insulin for 5 minutes. IRS-1 was immunoprecipitated from LDM samples (500μg), resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies (pY99). (A) Representative immunoblot. (B) Total IRS-1 immunoprecipitated in (A). (C) Results represent MEAN±SE of 3 independent experiments represented in (A). The insulin treated control was assigned the value 1.0 (Basal; Control 0.06±0.03, High G/I 0.22±0.08, High G/I + NAC 0.03±0.01 p = NS. Insulin; Control 1.0, High G/I 1.03±0.35, High G/I + NAC 0.89±0.32, p = NS).
The p85 subunit of PI3K binds to tyrosine phosphorylated IRS-1. Therefore, the amount of p85 associated with IRS-1 was determined. IRS-1 was immunoprecipitated from the LDM, resolved by SDS-PAGE and immunoblotted with p85 antibodies. In control cells, insulin increased the amount of p85 bound to IRS-1 approximately 5-fold above basal (Control: Basal 0.17±0.06, Insulin 1.0) (Fig. 3.14). Cells treated with High G/I displayed similar levels of p85 associated with IRS-1 in both basal and insulin treated cells (High G/I: Basal 0.25±0.19, Insulin 1.26±0.19, p = NS, n=3). In cells co-incubated with NAC and High G/I, the amount of p85 associated with IRS-1 in basal and insulin treated cells was similar to the control and High G/I treated cells (High G/I + NAC: Basal 0.30±0.19, Insulin 0.81±0.16, p = NS) (Fig. 3.14).

3.3.5 Effect of High G/I and NAC on IRS-1 Tyrosine Phosphorylation in Total Cell Lysates

IRS-1 tyrosine phosphorylation was measured in total cell lysates, since recent studies have suggested that disruption of IRS-1 function may vary among cellular compartments (115). In control cells, insulin increased tyrosine phosphorylation of IRS-1 (Control; Basal 0.04±0.02, Insulin 1.0) (Fig. 3.15). Cells treated with High G/I, displayed higher basal levels of tyrosine phosphorylation but lower levels after insulin treatment compared to the control. These differences did not reach statistical significance (High G/I: Basal 0.30±0.14, Insulin 0.65±0.11, p = NS, n=3). Cells co-incubated with High G/I and NAC displayed a tyrosine phosphorylation pattern similar to the control, in both basal and insulin treated cells (High G/I + NAC; Basal 0.15±0.08; Insulin 1.07±0.43, p = NS). Due to variability, tyrosine phosphorylation of IRS-1 in response to
FIG. 3.14 Effect of High G/I and NAC on IRS-1 associated p85 in the LDM. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC, respectively). Following the incubation, cells were washed and treated with 100nM insulin for 5 minutes. IRS-1 was immunoprecipitated from LDM samples (500μg), resolved by SDS-PAGE, transferred to nitrocellulose and probed with an anti-p85 antibody. (A) Representative immunoblot. (B) Results represent MEAN±SE of 3 independent experiments. The insulin treated control was assigned a value of 1.0 (Basal; Control 0.17±0.06, High G/I 0.25±0.19, High G/I + NAC 0.30±0.19, p = NS. Insulin; Control 1.0, High G/I 1.26±0.19, High G/I + NAC 0.81±0.16, p = NS).
FIG. 3.15 Effect of High G/I and NAC on IRS-1 tyrosine phosphorylation in total cell lysates. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC, respectively). Following the incubation, cells were washed and treated with 100nM insulin for 5 minutes. IRS-1 was immunoprecipitated from cell lysates (1mg), resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies (pY99). (A) Representative immunoblot. (B) Results represent MEAN±SE for 3 independent experiments. The insulin treated control was assigned the value 1.0 (Basal; Control 0.04±0.02, High G/I 0.30±0.14, High G/I + NAC 0.15±0.08, p = NS. Insulin; Control 1.0, High G/I 0.65±0.11, High G/I + NAC 1.07±0.43, p = NS).
insulin was not significantly different among conditions (Fig. 3.15). 18 h treatment with NAC alone had no effect on insulin-stimulated IRS-1 tyrosine phosphorylation (Basal: Control 0.16, NAC 0.11, Insulin: Control 1.0, NAC 0.88). Total levels of IRS-1 in cell lysates were unchanged by the treatments (Control 1.0, High G/I 1.08±0.22. High G/I + NAC 1.01±0.15, p = NS, n = 3, NAC alone 0.78) (Fig. 3.16).
FIG. 3.16 Effect of High G/I and NAC on IRS-1 levels in total cell lysates. Adipocytes were incubated for 18 h in media containing 20mM glucose/100nM insulin in the absence and presence of 5mM NAC (High G/I and High G/I + NAC, respectively), or with 5mM NAC alone. Cell lysates (80µg) were resolved by SDS-PAGE, transferred to nitrocellulose and blotted with an anti-IRS-1 antibody. (A) Representative immunoblot. (B) Represents the MEAN±SE of 3 independent experiments. The control was assigned the value 1.0 (Control 1.0, High G/I 1.08±0.22, High G/I + NAC 1.01±0.15, p = NS, NAC; 0.78).
3.4 Discussion

Previous work in our laboratory has shown that isolated rat adipocytes chronically treated with high glucose/insulin, or glucosamine, displayed marked decreases in insulin-stimulated glucose uptake which could be prevented by NAC. The present study has showed that insulin resistance induced by high glucose/insulin or glucosamine is associated with impaired translocation of Glut4 to the plasma membrane, as reported previously (73). Co-incubation with NAC prevented the impairment in Glut4 translocation, suggesting that NAC prevents insulin resistance. The fact that NAC prevented both high glucose/insulin and glucosamine induced insulin resistance implies that the site of action of NAC is at a common point in the two pathways. In addition, the ability of NAC to prevent insulin resistance induced by high glucose/insulin or glucosamine suggests a potential role of oxidative stress in the induction of insulin resistance in rat adipocytes.

In contrast to Glut4 translocation, high glucose/insulin and glucosamine had different effects on Glut1 expression. High glucose/insulin decreased Glut1 levels in the plasma membrane. High glucose and insulin have shown to have independent effects on Glut1 expression. In cell cultures prolonged treatment with high glucose decreases Glut1 expression (27). El-Kebbi et al. (110) have shown that treatment of BC3H-1 cells, a cultured skeletal muscle cell line, for 24 h in 25mM glucose resulted in an 80% decrease in Glut1 content. Although chronic insulin treatment has been shown to increase Glut1 expression in L6 muscle cells and 3T3-L1 adipocytes (111,112), this effect was not seen
in isolated rat adipocytes (73). Therefore, it appears that high glucose has a dominant effect on the levels of Glut1 in rat adipocytes incubated with high glucose/insulin. Garvey et al. (73) have shown that high glucose and insulin together decreased Glut1 levels, but chronic insulin treatment alone had no effect suggesting that increased metabolism of glucose causes a reduction in Glut1 protein levels. In contrast to high glucose/insulin, glucosamine treatment, either in the presence or absence of NAC, had no effect on Glut1 protein levels. The difference between the effects of high glucose/insulin and glucosamine may be explained by the different point of entry into the hexosamine biosynthesis pathway by these agents. For high glucose to enter the hexosamine pathway, it first has to saturate glycolysis and glycogen synthesis before being converted to glucosamine-6-phosphate by GFA (75). Intermediates from any of these pathways could potentially be involved in the regulation of Glut1 levels (113). Glucosamine bypasses these steps and enters the hexosamine pathway distal to GFA (75). NAC was able to prevent the decrease in Glut1 induced by high glucose/insulin. How NAC prevents the decrease in Glut1 is not clear. NAC may be acting upstream of GFA thereby blocking the generation of the glucose signal. In contrast to the present study, Rudich et al. (104) have shown that prolonged oxidative stress, induced by incubating cells with micromolar concentrations of H₂O₂ increases Glut1 levels in 3T3-L1 adipocytes.

IRS-1 tyrosine phosphorylation in response to insulin was not altered with high glucose/insulin treatment whether measured in the LDM or in total cell lysates, suggesting that, in this model, there was no significant serine/threonine phosphorylation of IRS-1 preventing tyrosine phosphorylation. In addition, the association of p85 with IRS-1 in response to insulin was not altered in the LDM fraction. Due to technical
difficulties, IRS-1 associated p85 in total cell lysates was not measured. Since almost all of the IRS-1 is located in the LDM in rat adipocytes (36), the results obtained from the experiments examining IRS-1 in the LDM fraction should accurately reflect the status of these proteins under these conditions. Lima et al. (114), have shown that glucose/insulin induced desensitization of glucose transport in rat adipocytes occurred without effect on IRS-1 tyrosine phosphorylation. High glucose was also without effect on IRS-1 tyrosine phosphorylation and PI3K activity in rat skeletal muscle (79). In contrast, treatment of 3T3-L1 adipocytes with H2O2, inhibited insulin stimulated IRS-1 tyrosine phosphorylation and IRS-1 associated PI3K activity in the LDM (115).

Although not statistically significant, basal levels of IRS-1 tyrosine phosphorylation and association with p85 in high glucose/insulin, and high glucose/insulin + NAC, treated cells tended to be higher compared to control cells. One possibility for this effect could be that following the 18 h incubation there was some residual effect of insulin remaining after the washing steps (114).

In the high glucose/insulin model of insulin resistance, total levels of IRS-1 were unchanged. Some insulin resistance states have shown to display decreased levels of IRS-1 expression, for example, chronic insulin treatment of 3T3-L1 adipocytes led to a significant reduction in IRS-1 protein levels (117).

In addition to IRS-1, rat adipocytes also contain IRS-2, which is also located in the LDM fraction (36). IRS-2 binds to p85 and can mediate the action of insulin to stimulate Glut4 translocation (118). Therefore, it is possible that in rat adipocytes treated with high glucose/insulin, IRS-2 tyrosine phosphorylation in response to insulin may be
impaired. Anai et al. (36) have shown that in rats rendered insulin resistant by high fat feeding, expression of IRS-2, and to a lesser extent IRS-1, was reduced in adipose cells.

Akt1/PKBα activation by insulin was impaired in cells treated with high glucose/insulin which was prevented by NAC. The decrease in kinase activity with high glucose/insulin treatment was accompanied by decreased phosphorylation of Ser473 suggesting an upstream signaling defect. PI3K activity and lipid products were not measured in the present study, therefore the possibility remains that there might be a defect at this step. There may also be a defect in the upstream kinase that phosphorylates Ser473. Although not measured in this study, phosphorylation of Thr308 may also be reduced with high glucose/insulin treatment which would implicate defective PDK-1 activity. High glucose/insulin may activate protein phosphatase 2A (PP2A), a serine/threonine phosphatase which could dephosphorylate and inactivate Akt1/PKBα (62). Co-incubation with NAC prevented the defect in Akt1/PKBα activation and Ser473 phosphorylation suggesting that one point at which NAC is acting is upstream of Akt1/PKBα and downstream of IRS-1. A discordance between IRS-1/PI3K and Akt1/PKBα activation has previously been shown in ceramide induced insulin resistance. Summers et al. (119), have shown that exposing 3T3-L1 adipocytes to the short-chain ceramide analog, C2-ceramide, impaired insulin stimulated glucose uptake, Glut4 translocation, and Akt1/PKBα and Akt2/PKBβ activities. These defects occurred despite normal insulin stimulated IRS-1 tyrosine phosphorylation, association with p85, and PI3K activity.

High glucose has previously been reported to impair Akt1/PKBα activation in skeletal muscle (79). Hyperglycemia could impair Akt1/PKBα by a PKC dependent
mechanism (79). Barthel et al. (116) have shown that prior activation of PKC by PMA inhibited the subsequent ability of insulin to stimulate Akt1/PKBα and Akt3/PKBγ activities in 3T3-L1 fibroblasts and adipocytes. Hyperglycemia has been shown to activate PKC (86). Interestingly, H₂O₂, has also been shown to activate PKC (120). If oxidative stress is operative in the present high glucose/insulin model, this mechanism of Akt1/PKBα inactivation remains possible.

Impaired Akt/PKB activation has also been shown to occur in 3T3-L1 adipocytes treated with H₂O₂. Lipoic acid, an antioxidant like NAC, was able to prevent the impairment in Akt1/PKBα activation induced by H₂O₂. These findings suggest that Akt1/PKBα activation may be sensitive to the oxidation state of the cell.

Activation of Akt1/PKBα has been associated with the translocation of Glut4 to the plasma membrane in response to insulin. Insulin resistant cells (high glucose/insulin treated) displayed approximately 50% lower Akt1/PKBα activation than the control or NAC treated cells. How much of a reduction in activity is necessary to impair Glut4 translocation is not yet known. Wang et al. (41) compared various dominant-negative mutants of Akt1/PKBα on Glut4 translocation and showed that a 2-fold increase in Akt1/PKBα activity was enough to fully translocate Glut4 to the plasma membrane. In the present study, the resistant cells displayed a 7-fold increase in Akt1/PKBα activity above basal. Whether the decrease in Akt1/PKBα activity can account for the impairment in Glut4 translocation is not certain.

In addition to Akt1/PKBα, rat adipocytes also express Akt2/PKBβ (121). Most studies in the past have focused mainly on Akt1/PKBα. Recently, numerous studies have implicated Akt2/PKBβ in the stimulation of Glut4 translocation by insulin in rat
adipocytes (29,42). Preliminary data from our laboratory suggest that Akt2/PKBβ activation by insulin is not impaired with high glucose/insulin treatment.

Treatment with NAC alone led to a reduction in Akt1/PKBα activation by insulin with no change in Ser473 phosphorylation. Why NAC reduced Akt1/PKBα activation is presently not clear. There are two possibilities that may explain the discordance between Akt1/PKBα activation and Ser473 phosphorylation. First, full activation of Akt1/PKBα requires phosphorylation on both Ser473 and Thr308 (21). Phosphorylation on either residue alone results in only partial activation of the kinase (21). The possibility exists that phosphorylation on Thr308 may be defective. Second, the kinase assay may be a more sensitive measure than Ser473 immunoblotting.

There are several possibilities that may explain the different signaling defects observed between the present study, from those observed in 3T3-L1 adipocytes exposed to H₂O₂ (104,105,115). First, the cells used were different. Although both types represent adipocytes, the two cell types may respond differently to various treatments. For example, chronic incubation of 3T3-L1 adipocytes with insulin was able to markedly decrease insulin-stimulated glucose uptake (78), while having a minimal effect in isolated rat adipocytes (73). In addition, chronic incubation of 3T3-L1 adipocytes with glucose and insulin decreased Glut4 levels (78), something not seen in isolated adipocytes (73). Second, the method of generating reactive oxygen species was different between the two models of insulin resistance. In the H₂O₂ model, reactive oxygen species were generated in the media surrounding the cells, whereas the high glucose/insulin model suggests that reactive oxygen species are generated inside the cell from metabolism. Reactive oxygen
species generated outside the cell may activate different stress pathways. It is possible that cells may respond differently to various forms of oxidative stress.

Overall, while various models of insulin resistance have all shown impaired translocation of Glut4 to the PM in response to insulin, there have been no consistent signaling defects observed. One possibility may be that different factors which induce insulin resistance generate different signals resulting in differential defects in insulin signaling. Another possibility may be the duration of the treatments. Some defects may occur early on, while others take longer to manifest.

To summarize and conclude, NAC, an antioxidant and glutathione (GSH) precursor, was able to prevent the impairment in Glut4 translocation induced by high glucose/insulin and glucosamine. NAC was also able to prevent the decrease in Glut1 expression and Akt1/PKBα activation and phosphorylation observed in cells treated with high glucose/insulin. These findings suggest a role for oxidative stress as a potential mechanism in the induction of insulin resistance in this model. In addition, these findings are relevant for Type II diabetic patients where hyperglycemia and hyperinsulinemia are likely present and may worsen peripheral insulin resistance. Finally, NAC may provide the potential design for novel therapeutic agents.
GENERAL DISCUSSION

The studies described in this thesis (Chapters 2 and 3) were designed to investigate the potential role of Akt1/PKBα in the stimulation of glucose uptake and Glut4 translocation using 2 different models. In the first model (Chapter 2) using insulin, vanadate, and pV to stimulate glucose uptake, the data support a role for Akt1/PKBα in Glut4 translocation mediated by insulin. Thus wortmannin pretreatment of cultured rat skeletal muscle cells (L6 cells) inhibited PI3K and subsequent activation of Akt1/PKBα by insulin in association with inhibition of insulin-mediated Glut4 translocation and glucose uptake (43). In the second model, further support for Akt1/PKBα in insulin stimulated Glut4 translocation came from the study of Akt1/PKBα in rat adipocytes rendered insulin resistant by treatment with high glucose/insulin. In these cells insulin stimulated Glut4 translocation and glucose uptake were impaired by 50% and 60% respectively. In these same cells, Akt1/PKBα activation by insulin was reduced by 44%. Importantly, co-incubation with NAC, which prevented the impairment in Glut4 translocation and glucose uptake, also corrected the defect in Akt1/PKBα activation. The close association between these parameters in both models strongly supports a role for Akt1/PKBα in mediating insulin induced Glut4 translocation and subsequent glucose uptake. Chronic treatment of rat adipocytes with glucosamine also resulted in impaired Glut4 translocation and glucose uptake stimulated by insulin. Co-incubation with NAC prevented these defects. The activation of Akt1/PKBα in this third model has not yet been studied but will be important to determine. These data strongly suggest but do not prove that the Akt1/PKBα defect is responsible for the insulin resistance of glucose uptake.
transport. To further examine this question, it would be worthwhile to activate Akt1/PKBα in these insulin resistant cells. This could be accomplished by using okadaic acid (56) or by transfecting a constitutively active form of the enzyme (38-40). The effects on Glut4 translocation could then be observed. To date, a specific inhibitor of Akt1/PKBα has not been found (22). The majority of studies implicating Akt1/PKBα in Glut4 translocation have used constitutively active or dominant negative forms of the enzyme. The development of a specific pharmacological inhibitor would also contribute significantly to our understanding of the role of this enzyme in the stimulation of Glut4 translocation by insulin.

In these studies the site of the glucose transport defect in the insulin resistant (High G/I treated) adipocytes appears to lie at least in part at the level of Akt1/PKBα. However, the cause of the defect in this enzyme is not yet clear. Thus, insulin-stimulated tyrosine phosphorylation of IRS-1 and the association of IRS-1 with the p85 regulatory subunit of PI3K was found to be normal. In general, this association implies an intact ability of insulin to stimulate PI3K and generate the lipid second messengers PtdIns-3,4-P2 and PtdIns-3,4,5-P3. The steps between PI3K and Akt1/PKBα include activation of PDK1 and PDK2 which are immediately downstream of PI3K and upstream of Akt1/PKBα. Future studies must determine whether these enzymes are normally activated in response to insulin or are defective in the insulin resistant adipocytes. Another possibility to explain such a defect is increased activity of a lipid phosphatase such as SHIP (SH2-domain containing inositol 3' phosphatase). This could potentially result in a reduction in the active PI3K lipid products in the face of normal PI3K activity.
Finally, the data presented here (Chapter 2) using vanadate and pV implicate that at least one novel pathway independent of PI3K and Akt1/PKBα exists by which glucose uptake and Glut4 translocation may be stimulated.
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December 17, 1999

To Whom It May Concern,

I am writing to seek permission for my student Elena Bogdanovic to republish figures originally published in our article in Diabetes in her M.Sc. thesis. The figures in question are Fig. 8 and Fig. 9 found in Tsian, E., Bogdanovic, E., Sorisky, A., Nagy, L., Fantus, I.G. "Tyrosine Phosphatase Inhibitors, Vanadate and Pervanadate, Stimulate Glucose Transport and GLUT Translocation in Muscle Cells by a Mechanism Independent of Phosphatidylinositol 3-kinase and Protein Kinase C", Diabetes 47:1676-1680, 1998.

Thank you for your consideration of this request.

Sincerely,

George Fantus, M.D., F.R.C.P.(C)

600 University Avenue, Suite 780, Toronto, Ontario M5G 1X5