April 2005

Effects of GAPDH, PGK and PGAM on Insulin-Stimulated Glucose Transport in 3T3-L1 Adipocytes

Aaron Raphael Grinstein

Worcester Polytechnic Institute

Follow this and additional works at: https://digitalcommons.wpi.edu/mqp-all

Repository Citation
Effects of GAPDH, PGK and PGAM on Insulin-Stimulated
Glucose Transport in 3T3-L1 Adipocytes

A Major Qualifying Project Report
Submitted to the Faculty of the
WORCESTER POLYTECHNIC INSTITUTE
in partial fulfillment of the requirements for the
Degree of Bachelor of Science
In
Biochemistry
by

Aaron Grinstein
April 28, 2005

APPROVED:

Mike Czech, Ph.D.
Program in Molecular Medicine
Umass Medical Center
Major Advisor

David Adams, Ph.D.
WPI Project Advisor
Glucose transporter 4 (GLUT4) moves from perinuclear storage regions to the plasma membrane in response to insulin and facilitates glucose uptake. This MQP examined the roles of three proteins (GAPDH, PGK, and PGAM) in glucose uptake and GLUT4 trafficking in 3T3-L1 adipocytes by performing glucose uptake assays on adipocytes in which these proteins were selectively knocked down using RNAi. GLUT4 trafficking was visualized by transfection with Myc-tagged GLUT4-GFP and immunofluorescence. The data indicate no effect by PGK, while PGAM and GAPDH inhibited both glucose uptake and GLUT4 fusion to the plasma membrane. GAPDH may be required for general cellular secretion pathways.
# TABLE OF CONTENTS

- Signature Page ................................................................. 1
- Abstract .............................................................................. 2
- Table of Contents .............................................................. 3
- Acknowledgements .............................................................. 4
- Background ......................................................................... 5
- Project Purpose ................................................................. 16
- Methods ............................................................................. 17
- Results ............................................................................... 23
- Discussion ......................................................................... 31
- Bibliography ....................................................................... 33
ACKNOWLEDGEMENTS

I would like to thank Michael Czech for the use of his laboratory and for all his help with data analysis. I would also like to especially thank Jin Gyoong Park, who provided me with key mentorship and advice, including help with various techniques and the design of several experiments. I must also thank Sabina Semiz, John Holik and Shraddha Patel for their assistance with experimental processes in the lab, as well as Paul Furcinitti, who provided extensive help with the microscopy and digital imaging of the project. Sabina Semiz and Jin Gyoong Park also supplied me with several buffers and reagents needed to complete certain experiments. Finally, I would like to thank David Adams for helping to initiate the project, and for all of his feedback and support in designing and writing this report.
BACKGROUND

Diabetes

Over 18 million people in the United States suffer from diabetes, and about one million new cases are diagnosed each year. Diabetes is the sixth leading cause of death in this country (responsible for 73,000 deaths in 2002), and it results in approximately 132 billion dollars annually in direct and indirect medical costs (Centers for Disease Control and Prevention, 2005). The substantial morbidity and mortality caused by this disease, along with its enormous economic burden, make it a major public health concern. Medical advances that can delay the onset and slow the progression of diabetes are desperately needed to alleviate the health care costs and improve the living conditions associated with the disease (Ray et al., 1998).

Diabetes is a disease in which the body does not properly produce or use insulin, a hormone required for the conversion of starches and sugars into energy. There are two types of diabetes, insulin-dependent diabetes mellitus (IDDM), or Type 1, and non-insulin-dependent diabetes mellitus (NIDDM), Type 2. Type 1 diabetes results from the destruction of pancreatic beta cells (insulin-producing cells) by the body’s immune system. Type 1 accounts for about 5-10% of all cases, usually occurring in juveniles and adolescents who become dependent on insulin injections to remain healthy. The more common form of diabetes is type 2, responsible for 90-95% of diabetes cases in the United States (American Diabetes Association, 2004).

In Type 2 diabetes the body cannot properly use insulin, resulting in the inability to regulate glucose homeostasis. This state is known as insulin resistance, in which the beta cells of the pancreas no longer produce the necessary amounts of insulin, or cells no longer respond to whatever amount of insulin secreted. Type 2 diabetes is usually found in adults over the age of 40, although its prevalence in younger adults has increased in recent years. The disease’s
symptoms mostly result from high blood glucose levels and cells being starved of energy, and may include extreme hunger/thirst, increased fatigue and blurry vision (Centers for Disease Control and Prevention, 2005). Over time the high blood sugar levels of diabetes can lead to severe complications, such as heart disease and stroke, and even death. Other complications include high blood pressure, blindness, kidney disease, nervous system disease, and limb amputations. Although the cause of diabetes is not known, it has been linked to genetics and environmental factors such as obesity and lack of exercise (American Diabetes Association, 2004). To fully understand the purpose of this MQP, a thorough understanding of the function and role of insulin is needed.

**Insulin**

Insulin is secreted by the beta cells of the Islets of Langerhans in the pancreas and is the principal hormone controlling blood glucose levels in mammals (Garrett and Grisham, 1999). It was first sequenced in the early 1950s by Frederick Sanger, whose work eventually made possible the production of unlimited amounts of human insulin (Rosenfeld, 2002). Insulin is produced by beta cells as proinsulin, a single-chain precursor, which enters the Golgi network and is proteolytically converted into the mature protein that consists of two polypeptide chains, 21 and 30 amino acids in length, linked by two disulfide bonds (Osterbye et al., 2001). The secondary structure of insulin is critical to its normal receptor binding and cellular signalling (Chang et al., 2003).

In response to rising blood glucose levels, insulin is secreted from the pancreas and stimulates glucose uptake and metabolism in muscle and adipose tissue (White and Kahn, 1994). It also induces synthesis of some glycolytic enzymes, such as glucokinase, phosphofructokinase
and pyruvate kinase, and inhibits gluconeogenesis by the liver. Overall insulin action allows humans to respond quickly to increasing blood glucose concentrations and maintain glucose homeostasis (Garrett and Grisham, 1999).

Insulin resistance, the main factor in type 2 diabetes, is a state in which cells no longer respond to normal circulating insulin levels. It leads to the inability of muscle and adipose cells to take in the necessary amount of glucose and, as a result, dangerously high blood glucose levels (Ducluzeau et al., 2002). The metabolic effects of insulin on target cells are highly regulated and specific, and they are mediated through the protein interactions of the insulin receptor. Understanding the signalling pathways used by this receptor will yield insight into the mechanisms of insulin resistance and non-insulin-dependent diabetes mellitus (Virkamäki et al., 1999).

**GLUT4**

Insulin plays an important role in many steps of glucose metabolism and one of its most important effects is its ability to increase the rate of cellular glucose uptake. It has been shown that insulin-stimulated glucose uptake is achieved through translocation of the glucose transporter isoform GLUT4 to the plasma membrane (Ducluzeau et al., 2002). While 11 facilitative hexose transporters have been previously identified, GLUT4 is the major insulin-responsive glucose transporter isoform and hence has become a major focus of diabetes research (Watson and Pessin, 2001). Expressed primarily in muscle and adipose tissue, GLUT4 helps to mediate a highly specialized glucose-transport system, which can be rapidly upregulated by insulin stimulation to allow these tissues to increase their rate of glucose transport by 10-40-fold.
within minutes. Dysfunctional glucose uptake into muscle and fat cells contributes to the onset of type 2 diabetes (Bryant et al., 2002).

GLUT4 continuously cycles between the plasma membrane and intracellular compartments. Under basal conditions, the majority of GLUT4 resides in perinuclear regions due to efficient endocytosis and intracellular sequestration (Watson and Pessin, 2001). The pool of intracellular GLUT4 molecules does not consist of a homogeneous population of vesicles, but rather two compartments with distinct protein content and insulin sensitivity (Figure 1). Electron microscopy in 3T3-L1 adipocytes has revealed multiple tubulo-vesicular structures in perinuclear regions and clathrin-coated pits near the plasma membrane, and immunofluorescence and subcellular fractionation have shown that GLUT4 vesicles continuously recycle between membrane and these endosomal compartments (Figure 1 upper part) (Ducluzeau et al., 2002). However, as much as 60% of the GLUT4 present in adipocytes resides in a distinct compartment that lacks endosomal markers, called the GLUT4 storage vesicle (GSV) pool (Figure 1 lower part). The GSV pool provides a reservoir of GLUT4 that is much more sensitive to stimulation by insulin than the recycling endosomal GLUT4 vesicles (Rea and James, 1997).

![Figure 1: GLUT4 Compartments](image-url)

Possible model by which insulin may stimulate GLUT4 translocation and cellular glucose uptake. GLUT4 is found in both a unique pool (GSVs) (lower part of diagram) as well as in the recycling endosomes (upper part). In the latter, it co-localizes with GLUT1 and transferrin receptors. (Ducluzeau et al., 2002)
The cytoskeleton plays an important role in GLUT4 vesicle trafficking and intracellular sequestering (Ducluzeau et al., 2002). An intact cytoskeleton may be required for optimal trafficking of GLUT4 from the GSV pool, as microtubule depolymerization disrupts GLUT4 translocation by approximately 40%. The cytoskeleton may not, however, facilitate movement of GLUT4 present on membranes of the endosomal pool. Microtubule depolymerization does not affect insulin-stimulated translocation of the transferring receptor, also located on these endosomal membranes (Fletcher et al., 2000). Microtubules and intermediate filaments of the cytoskeleton may function to maintain the perinuclear localization of GLUT4 vesicles. Treatment of 3T3-L1 adipocytes with nocodazole, a microtubule-disrupting agent, causes perinuclear GLUT4 to disperse to the cell periphery. Furthermore, inhibition of the activity of dynein, a microtubule-based motor that directs cellular organelles to juxtanuclear regions, caused dispersion of perinuclear GLUT4 and significantly inhibited insulin-stimulated translocation of GLUT4 to the plasma membrane (Guilherme et al., 2000). It is becoming increasingly apparent that cytoskeletal integrity is important in maintaining perinuclear GLUT4 compartments and the insulin sensitivity of these GLUT-4 containing membranes.

Insulin causes a dramatic redistribution of GLUT4 to the plasma membrane, increases exocytosis of GLUT4, and may even slightly inhibit endocytosis, altogether greatly increasing cellular glucose uptake (Watson and Pessin, 2001). It has been shown that insulin utilizes multiple signalling pathways to recruit GLUT4 vesicles to the cell surface, and the different signalling cascades may recruit GLUT4 from different intracellular pools (Simpson et al., 2001). The insulin transduction pathway has been researched extensively, and many key proteins have been identified, but various downstream targets remain unknown, spurring further research (Thurmond and Pessin, 2001).
The Insulin Signalling Pathway

Insulin stimulates GLUT4 translocation to the plasma membrane and glucose uptake by binding its receptor to initiate a complex, multi-step signalling cascade (Figure 2) (Thurmond and Pessin, 2001). The insulin receptor (IR) is a ~350kDa transmembrane glycoprotein with intrinsic protein tyrosine kinase activity. When activated by serum insulin, the IR undergoes a conformational change and phosphorylates the insulin receptor substrate (IRS) family of proteins on multiple tyrosine residues (Virkamäki et al., 1999). Tyrosine phosphorylation of these proteins creates recognition sites for downstream effector proteins with SH2 and PTB binding domains (Watson and Pessin, 2001).

![Figure 2: PI3-Kinase Signalling](image)

Schematic model of insulin-regulated PI3-K signalling. Activation of the insulin receptor induces the tyrosine phosphorylation of the IRS family of docking proteins, which in turn engages PI3-K. The activation of PI 3-K leads to GLUT4 trafficking and exocytosis. (Watson and Pessin, 2001)

Phosphorylated IRS proteins provide docking sites for p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3-K), as well as Grb2. Grb2 upon docking causes the subsequent activation of the serine/threonine kinase cascade known as the MAPK cascade, the essential signalling pathway for mitogenesis (Virkamäki et al., 1999). PI3-kinase is a key
component of the pathway mediating insulin’s metabolic effects (Figure 2). PI3-K generates the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which functions to activate phosphoinositol-dependent protein kinase (PDK). PDK has been shown to phosphorylate and activate protein kinase B (PKB, also known as Akt) and two members of the protein kinase C family (PKCζ and PKCλ). These three kinases, activated in this PI3K-sensitive manner, have been reported to increase glucose transport and GLUT4 localization to the plasma membrane. Evidence shows that at least one isoform of Akt (Akt2/PKBβ) is absolutely required for insulin-stimulated glucose transport and GLUT4 translocation (Zhou et al., 2004). However, other studies have shown that inhibition of PKCζ/λ has no significant effect, and it remains unclear whether they are necessary downstream targets for regulation of GLUT4 by insulin (Watson and Pessin, 2001).

Research has revealed the importance of PI3-kinase in the insulin signalling cascade leading to glucose uptake and GLUT4 trafficking, through the use of dominant negative-mutants of PI3-K and pharmacological inhibitors such as wortmannin and LY294002 (Somwar et al., 2001). Inhibition of PI3-K activity by the metabolite wortmannin almost totally abolishes insulin-stimulated glucose uptake and inhibits translocation of GLUT4 vesicles to the plasma membrane (Virkamäki et al., 1999). Stably expressing active downstream targets of PI3-K, such as Akt, restored glucose transport and GLUT4 at the cell surface, showing that PI3-K requires downstream effectors and is not directly involved in GLUT4 transport (Hausdorff et al., 1999).

Studies have found that there must be at least one additional GLUT4-mediating insulin receptor signalling pathway that functions independent of PI3-K (Figure 3) (Watson and Pessin, 2001). Another substrate found to be tyrosine phosphorylated by the insulin receptor is the proto-oncoprotein Cbl, which associated with the IR through the Cbl-associated adapter protein
Upon activation, the CAP-Cbl complex dissociates from the IR and binds the caveolar protein flotillin, relocalizing to lipid raft subdomains of the plasma membrane (Baumann et al., 2000). Phosphorylated Cbl recruits the adaptor protein CrkII, which forms a complex with the guanylnucleotide exchange factor C3G through its SH2 domain. C3G acts on TC10, a Rho family GTPase that associates with lipid raft domains, converting it to its active, GTP-bound state (Ducluzeau et al., 2002). TC10 activation through the insulin-induced CAP/Cbl/CRKII/C3G complex leads to translocation of GLUT4 vesicles from the GSV pool, but the molecular targets of TC10 remain unknown. Nonetheless, evidence has revealed that the lipid raft compartmentalization of this signalling pathway is necessary for TC10 regulation of GLUT4 trafficking, and that it functions independently of the PI3-K pathway (Watson et al., 2001).

Figure 3: TC10 Activation
Schematic model of insulin-regulated TC10 activation. In addition to the activation of the PI 3-kinase pathway, a portion of the cell surface insulin receptor is thought to reside within the flotillin/caveolin-enriched lipid raft domains. (Watson and Pessin, 2001)

In addition to the two aforementioned pathways, there is yet another participant in the metabolic signalling of insulin. In a less-studied pathway, protein kinase C β (PKCβ) is
activated by diacylglycerol and inositol 1,4,5-trisphosphate (IP3), the effect of latter coming through release of internal calcium stores. Both PKCβ effectors are generated through PIP2 cleavage catalyzed by phospholipase Cγ (PLCγ), which functions in a PI3-K-dependent manner (Lorenzo et al., 2002). Insulin induces an increase in PKCβ translocation and phosphorylation, and selective inhibition of PKCβ by GF109203X diminishes insulin-stimulated glucose transport by ~20%, implicating the PLCγ-PKCβ pathway as another regulator of GLUT4 (Wright et al., 2003).

**Figure 4: Signalling Pathways Implicated in Insulin-Regulated Glucose Transport**

By selectively depleting expression of the intermediate proteins in these pathways, using siRNA, and examining glucose uptake and GLUT4 trafficking, studies suggest that the boxed pathway is required for regulation of GLUT4 by insulin. (Zhou et al., 2004)

Three signalling circuits have now been implicated in GLUT4 regulation in muscle and adipose tissue (Figure 4). However, these processes are as yet not fully understood. GLUT4 trafficking involves multiple proteins that sort and transport GSVs along membranes and cytoskeletal tracks, along with proteins that assist in vesicle fusion at the plasma membrane, and various components of these systems are still unidentified (Zhou et al., 2004). A novel research technique, known as small interfering RNA (siRNA)-mediated gene silencing, can be used to selectively knockdown individual proteins of target cells. The results of these experiments yield
insight into the role and function of target proteins (Timmons, 2002). RNAi-based gene silencing has already made possible the recognition of the required components of the insulin signalling pathways (see Figure 4), and it may help to identify the downstream targets of these and other metabolic pathways (Zhou et al., 2004; Jiang et al., 2003).

**GAPDH, PGK and PGAM**

Glucose uptake in adipose tissue via insulin-responsive GLUT4 is regulated by complex mechanisms involving both signalling and intracellular membrane trafficking. Our understanding of this system is incomplete, and it is likely to contain many unknown components (Czech and Corvera, 1999). In an effort to discover several of these unknown components, this project examined the effects on insulin-stimulated glucose transport of 3 proteins: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK) and phosphoglycerate mutase (PGAM). Research has implicated roles for these glycolytic enzymes in insulin signalling and microtubule-associated vesicle trafficking.

Studies have revealed that GAPDH displays diverse roles unrelated to its glycolytic function, including membrane fusion, cytoskeleton modulation and phosphotransfer activity, among others. These intracellular activities are all essential to the maintenance of membrane trafficking (Sirover, 1999). GAPDH was recently shown to be required for transport in early secretory pathway, including ER to Golgi traffic, through microtubule dynamics. It was also shown to be phosphorylated by atypical PKCs, proven downstream effector proteins of the insulin receptor signalling cascade (Tisdale, 2002). This evidence of phosphorylation by PKCs and involvement in membrane trafficking suggest that GAPDH may function in GLUT4 regulation.
as well. This project aimed to discover the role, if any, of GAPDH in the glucose transport mediated by GLUT4.

Another glycolytic enzyme, phosphoglycerate kinase (PGK), is known to exist in a functional complex with GAPDH (Ikemoto et al., 2003; Laschet et al., 2004). It is possible that the activities of GAPDH explained previously are regulated by the bound PGK. An additional goal of this project was to determine any effects PGK may have on GLUT4 modulation, whether directly or indirectly through GAPDH.

Phosphoglycerate mutase (PGAM), along with other glycolytic enzymes, was shown to be phosphorylated by epidermal-growth-factor-receptor (EGFR) kinase, and may play a role in the signalling pathway through the EGFR (Reiss et al., 1986). It was also demonstrated to be an effective substrate of the insulin receptor and phosphorylated by its tyrosine kinase activity (Sale et al., 1987). These data suggest PGAM may play a role, possibly through phosphotransfer activity, in various signalling cascades, including the insulin receptor signalling processes. The third and final objective of this MQP was to investigate the function, if any, of PGAM in GLUT4-mediated glucose transport. This research may provide insight into our still fragmentary understanding of insulin’s metabolic signalling pathways. It may, in the end, help to advance our knowledge of the physiology of the debilitating disease, diabetes.
PROJECT PURPOSE

The goal of this project was to gain further insight into the metabolic signalling pathway of insulin. Insulin resistance, a state in which muscle and adipose cells no longer respond to normal circulating levels of the hormone, results in the failure to regulate glucose homeostasis and is the main factor causing non-insulin-dependent diabetes mellitus. NIDDM (type 2 diabetes) comprises over 90% of all diabetes cases and is a major public health concern in the United States, and medical advances that can alleviate the suffering and health care costs of the disease are desperately needed. The mechanisms of diabetes lie in insulin-induced glucose metabolism, which is mediated by the insulin receptor. The insulin receptor initiates multiple signalling cascades that, through numerous protein interactions, recruit GLUT4 vesicles to the plasma membrane to enhance glucose uptake.

Complex systems regulate the signalling and intracellular membrane trafficking of GLUT4, and many components of these processes still unknown. Identification of the elusive downstream elements of the insulin signalling pathways is an important step toward fully understanding type 2 diabetes. Three proteins that have been implicated as possible effectors of GLUT4 regulation are the glycolytic enzymes GAPDH, PGK and PGAM. The objective of this MQP was to determine the effects, if any, of these proteins on insulin-stimulated glucose transport in adipocytes.
METHODS

Tissue Culture

Murine 3T3-L1 fibroblasts were cultured in 37°C incubators with 10% CO₂. The cells were maintained in complete low glucose media, Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Streptomycin and Penicillin, until 3 days after growing to confluence. The fibroblasts were then differentiated (Day 0 adipocytes) by incubation for 72 hours in complete DMEM with 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 mM dexamethasone and 1 mM insulin. The Day 3 adipocytes were then kept in complete DMEM without differentiation hormones until use.

Myc-GLUT4-GFP and siRNA Transfection

Myc-tagged GLUT4-GFP DNA and GAPDH, PGK and PGAM siRNA were transfected into adipocytes using electroporation. 3T3-L1 cells were differentiated on 150 mL Maxi plates and cultured for 4 days after differentiation. Day 4 adipocytes were washed twice with 1% phosphate buffered saline (PBS), and incubated in 4 mL of 1X trypsin for 5-10 minutes, until the cells visibly separated from the plates. 10 mL complete media was added to transfer the cells to a 15 mL Falcon centrifuge tube. The cells were centrifuged for 5 minutes at 1,000 rpm. The pellet was then washed with 10 mL complete media and centrifuged for 5 minutes at 1,000 rpm, two times. Next, the pellet was resuspended in 2 mL PBS and separated equally into 4 gene pulser cuvettes with 0.4 cm electrode gaps. The different solutions, described below, were mixed thoroughly and electroporated in a BioRad Gene Pulser II with a voltage of 0.18 kV and capacitance of 950 µF. After electroporation, 1 mL of complete media was quickly added to each cuvette, and they were incubated at 37°C for 10 minutes.
For the glucose uptake assays, each of the 4 cuvettes contained 15 µL of siRNA designed to specifically knockdown the expression of one of the proteins GAPDH, PGK or PGAM. The fourth cuvette contained 15 µL of scrambled siRNA, as a positive control. After the electroporation process, the transfected cells were transferred to 24-well plates. From each cuvette, 900 µL was transferred evenly into 9 different wells, each containing 1 mL of complete medium. 300 µL from each cuvette was added to 2 mL complete medium in 1 well of a 6-well plate, to be used to measure protein knockdown. The plates were then incubated at 37°C for 48 hours.

For the immunofluorescence, the cuvettes contained 4 distinct 15 µL siRNA solutions as per the glucose uptake experiment, along with 4 µL of the Myc-tagged GLUT4-GFP DNA construct. After electroporation, 900 µL from each cuvette was transferred evenly into 4 wells of a 6-well plate, each containing 2 mL complete medium and a glass collagen-coated coverslip. Again to measure protein knockdown, 300 µL from each cuvette was added to 2 mL complete medium in 1 well of a 6-well plate. Finally, the transfected adipocytes were incubated at 37°C for 48 hours.

**Glucose Uptake Assays**

3T3-L1 adipocytes transfected with GAPDH, PGK, PGAM and scrambled siRNA were starved for 3 hours with 500µL/well KRH buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.33 mM CaCl₂, 12 mM HEPES, pH 7.4) to achieve basal states. During the final 30 minutes of starvation, one third of the samples (3 wells from each set on the 24-well plates) were stimulated with 100 nM insulin, and 10 µM cytochalasin B was added to another third, in order to correct for non-specific uptake. Radioactively-labeled [³H] 2-deoxyglucose (2-DOG) was diluted 1:20
in cold 5.1 mM 2-DOG and kept on ice, and at the end of starvation, 10 µL was added to each sample for 5 minutes (final concentration 0.10 mM). The reaction was terminated by dumping the contents of the wells and washing the cells 3 times quickly with 1 mL ice cold KRH. The cells were then lysed with 0.4 mL 1% Triton-X-100 and radioactivity was measured in an LKB scintillation counter.

**Immunostaining and Microscopy**

Collagen-coated coverslips were used to help ensure that cells remained fixed during the agitation of the washing processes. The coverslips containing cells transfected with siRNA and GLUT4-GFP DNA were first starved for 3 hours in starvation media (DMEM with 1% FBS and 1% Streptomycin and Penicillin), with or without 100 nM insulin during the final 30 minutes. They were then washed twice in cold PBS, fixed with 3.7% Formaldehyde in PBS for at least 20 minutes, and washed twice in P-buffer (PBS with 1% FBS). Next, coverslips were incubated with primary mouse anti-Myc antibody diluted in P-buffer for 2 hours at room temperature and washed 5 times for 3 minutes in P-buffer. They were incubated with secondary Rhodamine-conjugated goat anti-mouse antibody diluted in P-buffer for 30 minutes at room temperature in the dark, and then washed again as before. Coverslips were mounted onto glass slides using DABCO and nail polish and stored at 4°C.

Fluorescence microscopy was carried out with an Olympus IX70-inverted light microscope with a Roper Scientific high resolution, thinned, back-illuminated charge-coupled device (CCD) digital camera, a Sutter filter wheel and shutter, and a PZT piezoelectric focus drive. These devices were controlled by METAMORPH image acquisition and analysis software. The cells were viewed using FITC and Rhodamine filters at 100X magnification, to
visualize the GLUT4-GFP conjugate in a green color and the Myc tag in red, when present outside the cell membrane. Pictures were taken with the microscope focused on a level with the middle of the cells, the point where stained molecules at the edges of the cell most clearly defined the plasma membrane.

**GAPDH Inhibition by Iodoacetate**

Day 4 adipocytes were reseeded onto 24-well plates, and after 24 hours were starved under the same conditions as the previously described glucose uptake assays. However, in this experiment, cells were treated with 0 µM, 10 µM, 100 µM, or 1 mM iodoacetate, for 30 minutes or 3 hours, under basal or insulin-stimulated conditions. Again, cytochalasin B was used in order to measure the non-specific uptake. Cells were lysed and radioactivity was measured as per the other 2-DOG assays.

**Adiponectin Secretion**

After electroporation, adipocytes transfected with only the protein-specific or scrambled siRNAs were transferred to a 24-well plate and incubated at 37°C for 24 hours. They were then starved overnight in starvation media, and the following morning half of the cells were stimulated with 100 nM insulin for 30 minutes. At the end of stimulation, the media was transferred to eppendorf tubes and protein concentrations were measured using a BSA reagent assay, in order to normalize the samples to the lowest protein concentration. SDS-PAGE and Western blots were carried out as described below, using α-ACRP30 primary antibody to detect adiponectin levels in the medium.
Cell Lysis

In order to measure the protein expression levels (and hence siRNA effectiveness) in the transfected cells, the cells were lysed 48 hour after transfection using a 1.5X lysis buffer. The buffer consisted of 20 mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA, 1mM ESTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 100 mM PMSF, 1 mM sodium o-vanadate, 1 mM aprotinin, and 1 mM leupeptin. The 6-well plates were placed on ice, washed twice with PBS, and incubated in lysis buffer for 10 minutes. Cells were scraped off each well and kept in eppendorf tubes on ice. The cells were sonicated 10 times and were centrifuged for 15 minutes at 13,000 rpm. The protein concentrations of the LDM supernatants were measured using a BSA reagent assay. Samples were then loaded with equal concentrations for the SDS-PAGE.

SDS-PAGE

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used in separate experiments to measure protein concentrations in siRNA-transfected cells and adiponectin secretion. Mini gels were cast with 10% Acrylamide and a 4% Acrylamide stacking gel. Samples were denatured in 100°C water for 1 minute, then loaded at equal concentrations into the stacking gel wells with a loading buffer consisting of 30% Glycerol, 25% 0.5 mM Tris, pH 6.8, 25% H2O, 12.5% 2-mercaptoethanol, 7.5% sodium dodecyl sulfate (SDS), and brilliant blue. Gels were run at 50 V until samples passed the stacking gel, then 150 V to completion, in a running buffer consisting of 82% H2O, 0.5% SDS, 3% Tris, and 14.5% glycine. Gel proteins were then transferred onto nitrocellulose membrane (2 hours at 150 mA) in a transfer buffer of 77.6% H2O, 20% MeOH, 0.1% SDS, 0.3% Tris, and 2% glycine.
Western Blots

The nitrocellulose membranes obtained from the SDS-PAGE were blocked with 5% skim milk in Tris-buffered saline with 0.5% Tween-20 (TBST) for 1 hour at room temperature or overnight at 4°C. They were next incubated with the appropriate primary antibodies diluted in 0.5% TBST for 2 hours at room temperature, and then washed 3 times for 10 minutes in TBST with gentle shaking. After washing they were incubated with the corresponding HRP-conjugated anti-goat, anti-mouse or anti-rabbit secondary antibodies, diluted in TBST, for 30 minutes at room temperature. Again the membranes were washed 3 times for 10 minutes in TBST, and proteins were detected using Western Lighting Chemiluminescence reagents.
RESULTS

The goal of this project was to determine the effects of the proteins GAPDH, PGK and PGAM on glucose transport in 3T3-L1 adipocytes. RNAi-mediated gene silencing was utilized to selectively knockdown the target proteins, and iodoacetate to inhibit GAPDH activity. Glucose uptake was measured in transfected cells, and GLUT4 trafficking was visualized by immunofluorescence. Adiponectin secretion levels were also measured.

Protein Knockdown by RNAi

Small interfering RNAs (siRNAs) were used to selectively deplete the expression levels of the target proteins, through mediation of the mRNAs of the genes encoding these proteins. To measure the effectiveness of the siRNAs, protein concentrations were measured 48 hours after transfection using cell lysates and blotting with appropriate antibodies (see Figures 5 and 6).

The top band present in every lane is a generic protein blot for PLC, in order to see whether the siRNAs had any toxic effects on the transfected cells. For both experiments (Figures 5 and 6), the bands located at ~135 kDa shows no difference in PLC concentration between the knockdowns and scrambled siRNA controls. While the shock of electroporation may have caused some cell death, these Western blots show that depleting these individual glycolytic enzymes was not fatal to these 3T3-L1 adipocytes.

In both blots (Figures 5 and 6), the samples in Lane 2 are from cells transfected with GAPDH siRNA. The band present at ~40 kDa shows 50-60% depletion of GAPDH in knockdown cells. Lane 4 contains PGK siRNA-transfected cell samples, and the band at ~50 kDa shows near 50% reduction in PGK levels. Finally, Lane 6, with the PGAM band present at ~30 kDa, indicates greater than 70% knockdown of PGAM expression.
Figure 5: Protein Knockdown in 2-DOG Uptake Experiments
Cells transfected with siRNA and used for 2-DOG uptake assays were lysed, and LDM fractions were used for SDS-PAGE and Western blotting, according to the methodology described in the previous section. Developed membranes are shown above with corresponding antibodies indicated in margins. Lanes 1, 3, 5 – scrambled siRNA, Lane 2 – GAPDH, Lane 4 – PGK, Lane 6 – PGAM.

Figure 6: Protein Knockdown in Immunofluorescence Experiments
Cells transfected with siRNA and Myc-GLUT4-GFP and used for immunofluorescence were lysed, and LDM fractions were used for SDS-PAGE and Western blotting, according to the procedures described previously. Developed membranes are shown above with corresponding antibodies indicated in margins. Lanes 1, 3, 5 – scrambled siRNA, Lane 2 – GAPDH, Lane 4 – PGK, Lane 6 – PGAM.
Western blot analysis clearly shows that the siRNAs were very effective in depleting target protein concentrations in 3T3-L1 adipocytes. They were not toxic to the cells, as seen by the equal PLC levels in experimental and control cells. They efficiently reduced expression levels of GAPDH, PGK and PGAM 50-70% 48 hours after transfection.

Inhibition of Glucose Uptake

![Graph showing glucose uptake](image)

**Figure 7: 2-DOG Uptake Assay using si-RNA-Transfected Cells**

Graph shows mean value ±SD from 2 experiments of total [3H] 2-deoxyglucose uptake compared to basal uptake of control cells transfected with scrambled siRNA. Cells were treated with or without 100 nM insulin for 30 minutes.

Glucose uptake was measured in cells transfected with scrambled, GAPDH, PGK, or PGAM siRNA as described in the Methods section. Figure 7 shows 2-deoxyglucose uptake values based on 100% uptake in control cells under basal conditions. Glucose transport in basal conditions was not affected by the 50-70% reduction of any of the three proteins. Insulin-stimulated uptake, however, was significantly inhibited by GAPDH and PGAM depletion (~50% by GAPDH, ~35% by PGAM). PGK-knockdown cells, on the other hand, showed only a small,
negligible decrease in insulin-stimulated glucose uptake. These data indicate that insulin-responsive glucose transport is in some way facilitated by GAPDH and PGAM, though not by PGK.

![Graph showing 2-DOG Uptake Assay using Cells Treated with Iodoacetate](image)

**Figure 8: 2-DOG Uptake Assay using Cells Treated with Iodoacetate**
Graph shows mean value ±SD from 2 experiments of total [³H] 2-deoxyglucose uptake compared to basal uptake of control cells not treated with iodoacetate. Cells were incubated with or without iodoacetate for 3 hours and with or without 100 nM insulin for 30 minutes.

GAPDH activity was inhibited by iodoacetate to further examine the protein’s effects on glucose transport. Figure 8 shows 2-deoxyglucose uptake, again based on 100% uptake of basal control cells, in cells treated with 0 µM, 100 µM, or 1 mM iodoacetate for 3 hours, under basal and insulin-stimulated conditions. Lower concentrations of iodoacetate and shorter incubation times (30 minutes) with iodoacetate displayed little or no effects (data not shown). Incubation for 3 hours in 100 µM or 1 mM iodoacetate nearly abolished insulin-stimulated uptake. 1 mM iodoacetate treatment also greatly inhibited basal glucose uptake.

The data presented in Figure 8 suggest that GAPDH plays an important role in glucose uptake. It is important to note, according to these results, that GAPDH may also be involved in
glucose transport in the basal state. While this experiment does not rule out the possibility of the protein’s involvement in insulin signalling, it implies that the function of GAPDH may lie in the basal trafficking of GLUT4, including endosomal membrane recycling, docking/fusion to the plasma membrane, endocytosis and/or exocytosis. No effect on basal uptake was seen in GAPDH-knockdowns (Figure 7), demonstrating that decreased levels of the protein may be sufficient to maintain the basal GLUT4 trafficking machinery. However, it may also be possible that 1 mM iodoacetate is toxic to the cells. This would explain the greatly decreased glucose uptake under both basal and insulin-stimulated conditions.

Inhibition of GLUT4 Translocation and Membrane Fusion

To look closer at the processes affected by GAPDH, PGK and PGAM, GLUT4 trafficking was examined. 3T3-L1 adipocytes transfected with siRNAs were also simultaneously transfected with Myc-GLUT4-GFP plasmid DNA, in order to visualize GLUT4 localization by immunofluorescence. Sample digital images taken using the methods and equipment described in the previous section are shown in Figure 9. Pictures in the same row of the figure are of the same cell using different filters.

Quantitative data from the microscopy experiments are shown in Figure 10. More than 50 adipocytes were counted per sample in each experiment, and the presence or absence of a red Myc rim, a green GFP rim, or both at the plasma membrane was determined for each cell. A cell exhibiting a Myc rim was defined as having greater than 50% of the cell surface (in the plane of the image) clearly marked by GLUT4 molecules in red. Cells with greater than 50% of the plasma membrane showing a green rim, or a large amount of associated GLUT4 molecules just beneath, were said to contain a GFP rim.
Figure 9: Immunostaining of Coverslips Containing Myc-GLUT4-GFP-Transfected Cells

Cells were transfected with the siRNA indicated to the left and Myc-GLUT4-GFP, and stimulated with 100 nM insulin for 30 minutes. The top row shows basal conditions. GLUT4 vesicles are visualized in green in the left column. Rhodamine-conjugated anti-Myc antibody is evident in red in the right column where GLUT4 has fused with the plasma membrane.
Figure 10: GLUT4 Translocation Measured by Immunofluorescence

Graphs represent data from 3 independent experiments with >50 3T3-L1 adipocytes counted per slide in each experiment. White bars represent cells under basal conditions, transfected with scrambled siRNA. (Left) Percentage of cells showing red Myc-GLUT4 rims, indicating fusion of GLUT4 to the plasma membrane through exocytosis. (Right) Percentage of cells showing GLUT4-GFP rims, or a large proportion of GLUT4 localized near the plasma membrane, indicating translocation of GLUT4 to the cell periphery.

Results from the immunostaining experiments are displayed in Figure 10 and are concurrent with results obtained from the glucose uptake assays. There was a ~35% decrease in cells with plasma membrane GLUT4-GFP in GAPDH-knockdown samples, while GFP rims in PGK- and PGAM-knockdowns were unaffected (Figure 10, left). GAPDH and PGAM depletion reduced the number of cells with plasma membrane Myc-GLUT4 ~60% and ~30%, respectively, while knockdown of PGK had no effect (Figure 10, right). Under basal conditions the proportion of cells containing GFP or Myc rims was very similar to control cells (scrambled), regardless of the siRNA used (data not shown).

These data indicate that GAPDH may be involved in either the insulin signalling pathway or the trafficking of GLUT4 vesicles, as depletion of the protein prevented normal translocation of GLUT4 to the cell periphery. However, as seen by the substantial decrease in plasma membrane Myc-GLUT4, GAPDH may also facilitate GLUT4 exocytosis. The data from Figure 10 (right) also implicates a role for PGAM in the exocytosis of GLUT4 vesicles.
Adiponectin Secretion Levels

To observe the effects of the protein knockdown on general secretion processes, particularly an insulin-responsive secretory pathway, levels of adiponectin secretion were measured. Figure 11 displays a Western blot containing the adiponectin band at ~35 kDa. Medium collected from scrambled siRNA-transfected cells showed approximately 100% increase in adiponectin. Knockdown of PGK had no effect in comparison to the control samples. Depletion of GAPDH caused a significant decrease (near 70%) in adiponectin secretion both with and without insulin stimulation. PGAM depletion, on the other hand, yielded a substantial increase in adiponectin levels in the medium, also in both basal and insulin-stimulated conditions (Figure 11, Lanes 7 and 8). These data suggest that GAPDH may be required for general cellular secretion and that PGAM may function to block general secretion.

<table>
<thead>
<tr>
<th>Insulin</th>
<th>Scr</th>
<th>GAPDH</th>
<th>PGK</th>
<th>PGAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 11: Adiponectin Secretion Levels of siRNA-Transfected Cells
Developed membrane of SDS-Page and Western blot of medium samples from cell transfected with the indicated siRNAs, blotted with adiponectin antibody. Cells were treated with or without 100 nM insulin for 30 minutes. Lanes 1,2 – scrambled siRNA, Lanes 3,4 – GAPDH, Lanes 5,6 – PGK, Lanes 7,8 – PGAM.
DISCUSSION

The data compiled in this project show significant inhibition of insulin-stimulated glucose uptake and GLUT4 membrane fusion in 3T3-L1 adipocytes with depleted levels of GAPDH or PGAM, along with decreased GLUT4 translocation in GAPDH knockdowns. PGK was shown to have no effect on any of these processes. Immunofluorescence data was crucial in concluding that PGAM’s mediating effects on glucose uptake are part of the exocytosis of GLUT4 vesicles, and that GAPDH functions in both GLUT4 exocytosis and the translocation of GLUT4 vesicles to the cell periphery.

The findings of this project show that GAPDH is important to the glucose transport system in adipocytes and are concurrent with prior research concerning GAPDH activity. GAPDH was previously shown to take part in membrane fusion, which may explain the decrease in GLUT4 plasma membrane fusion in GAPDH knockdowns. Furthermore, studies have also revealed that GAPDH facilitates organization of and transport along the cytoskeleton (Sirover, 1999). Since microtubules are important for intracellular compartmentalization and translocation of GSVs (Guilherme et al., 2000), it logically follows that GAPDH functions in the cytoskeletal transport of GLUT4. GAPDH may not function specifically in GLUT4 translocation and exocytosis, but rather in global secretory processes. This is evident in the diminished levels of adiponectin secretion of GAPDH knockdowns, and it agrees with previous findings regarding the role of GAPDH in the early secretory pathway (Tisdale, 2002). However, GAPDH was shown to be phosphorylated by PKCs downstream in the IR signalling cascade (Tisdale, 2002), and it may still be directly involved in the insulin signalling pathway regulating GLUT4 trafficking and membrane fusion. Future research will be necessary to determine the precise role of GAPDH in glucose transport, distinct from general secretion, in adipose tissue.
While GAPDH may facilitate global exocytosis, the results of this project suggest that PGAM may function specifically in GLUT4-related exocytic machinery. In contrast to GAPDH knockdowns, PGAM depletion caused an increase in basal and insulin-stimulated adiponectin secretion, indicating that PGAM may normally block general secretion processes. No prior research, however, has found PGAM to be a negative modulator of exocytosis. It is possible that knockdown of PGAM makes cells sick and leaky, and antibodies for another protein should be used alongside the blot for adiponectin as a loading control. Regardless of whether PGAM inhibits global secretion, it was shown to be phosphorylated by the insulin receptor (Sale et al., 1987) and clearly facilitates insulin-induced glucose uptake and GLUT4 membrane fusion. Additional research in the future may reveal which steps in GSV exocytosis are mediated by PGAM, or perhaps a downstream target of PGAM.

Type 2 diabetes is a major public health concern, and the quest to find potential drug targets continues today. The disease involves insulin resistance, caused by defects in the metabolic signalling cascade of insulin receptor. Previously identified and possible components of this pathway are being intensely researched. This project has provided an improved understanding of insulin signalling in adipose tissue, through further insight into the function of GAPDH, PGK and PGAM in the glucose transport system. Future work stemming from this research may expose the exact impact of these enzymes on insulin signalling and their specific role in diabetes.


Tisdale EJ (2002) Glyceraldehyde-3-phosphate Dehydrogenase is Phosphorylated by Protein Kinase C$\mu/\lambda$ and Plays a Role in Microtubule Dynamics in the Early Secretory Pathway. The Journal of Biological Chemistry. 277: 3334-3341.


