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Insulin-stimulated glucose uptake is a vital physiological process, which requires the translocation and fusion of insulin sensitive glucose transporter (GLUT4) vesicles from intracellular pools to the plasma membrane. Previous studies have implicated cortical actin reorganization in GLUT4-mediated glucose uptake. Not much is known, however, about how cortical actin is reorganized to allow GLUT4 vesicle fusion to the plasma membrane. A recent study found that myosin II is necessary for insulin-stimulated glucose uptake and implicated myosin II activity in cortical actin reorganization. Our study further examined the role of myosin II in insulin-stimulated glucose uptake. We found that myosin II associates with GLUT4 vesicles upon insulin stimulation. This study also found that myosin II is necessary for proper GLUT4 vesicle fusion and activation. This study is the first to demonstrate the dual role myosin II plays in GLUT4 vesicle fusion and activation during insulin-stimulated glucose uptake. Our results provide further understanding into cellular and molecular mechanisms necessary for proper glucose uptake.

THE ROLE OF MYOSIN II IN GLUT4 ACTIVITY AND MEMBRANE FUSION  
DURING INSULIN-STIMULATED GLUCOSE UPTAKE  
IN 3T3-L1 ADIPOCYTES

by

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Greensboro  
2006

Approved by

---

Committee Chair

To ...

My One True Love

Without Your support, I sink.

Without Your help, I drown.

Without Your love, I perish.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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## CHAPTER I

### INTRODUCTION

Once a disease only associated with a small percentage of the juvenile and elderly subpopulations, diabetes has become indiscriminate and affects individuals of every age, race, gender and socioeconomic group.

Because diabetes has begun to affect such a large and diverse group of people, the need to understand its pathology on the physiological, cellular, molecular, and genetic levels has become more urgent. The knowledge attained through these studies can then lead to the development of preventative measures, early detection and therapeutic treatments.

#### **Type 1 Diabetes and Type II Diabetes**

Within the last fifty years, the prevalence of diabetes among Americans has increased. In 1958, only 0.93% of the American population was diabetic (1). Currently, 120 million people worldwide are diabetic, 20.8 million of which are U. S. citizens (2,3). That is 7% of the U. S. population. If the occurrence of diabetes continues to increase, there will be a projected 300 million diabetics worldwide by 2025 (3).

There are two major types of diabetes: Type I and Type II (1). Type I diabetes or insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease in which the

insulin-producing beta cells of the pancreas are destroyed (4). Thus, Type I diabetics are required to take exogenous insulin (4).

Type II diabetes or non-insulin-dependent diabetes mellitus (NIDDM) is the most common form of diabetes, affecting approximately 90% of diabetics (2, 4). NIDDM is a result of insulin resistance, which leads to inefficient  $\beta$ -cell insulin production as well as the development of numerous health conditions, such as diabetic retinopathy (blindness), renal failure, hypertension, and myocardial infarctions (2, 4).

### **What is Diabetes?**

With the increasing occurrence of diabetes among the world's population, it is important to understand what diabetes is. In general, diabetes is characterized by high serum glucose levels and insufficient insulin production, secretion, or activity (4). In normal conditions, the rapid removal of glucose occurs when the hormone insulin signals adipocytes and skeletal muscle cells to clear excess serum glucose. Without insulin-stimulated glucose uptake, the majority of glucose remains in circulation for an extended period of time. Consistently elevated serum glucose levels lead to insulin resistance.

### **Understanding NIDDM**

Before the onset of NIDDM, individuals first experience insulin resistance. Insulin resistance results from an improper response of adipose tissue and skeletal muscle tissue to serum insulin leading to a decrease in glucose uptake (4). If insulin resistance remains untreated, NIDDM can develop.

In order to develop effective treatments and early intervention and prevention methods, it is necessary to understand the underlying cellular and molecular mechanisms that result in the development of NIDDM.

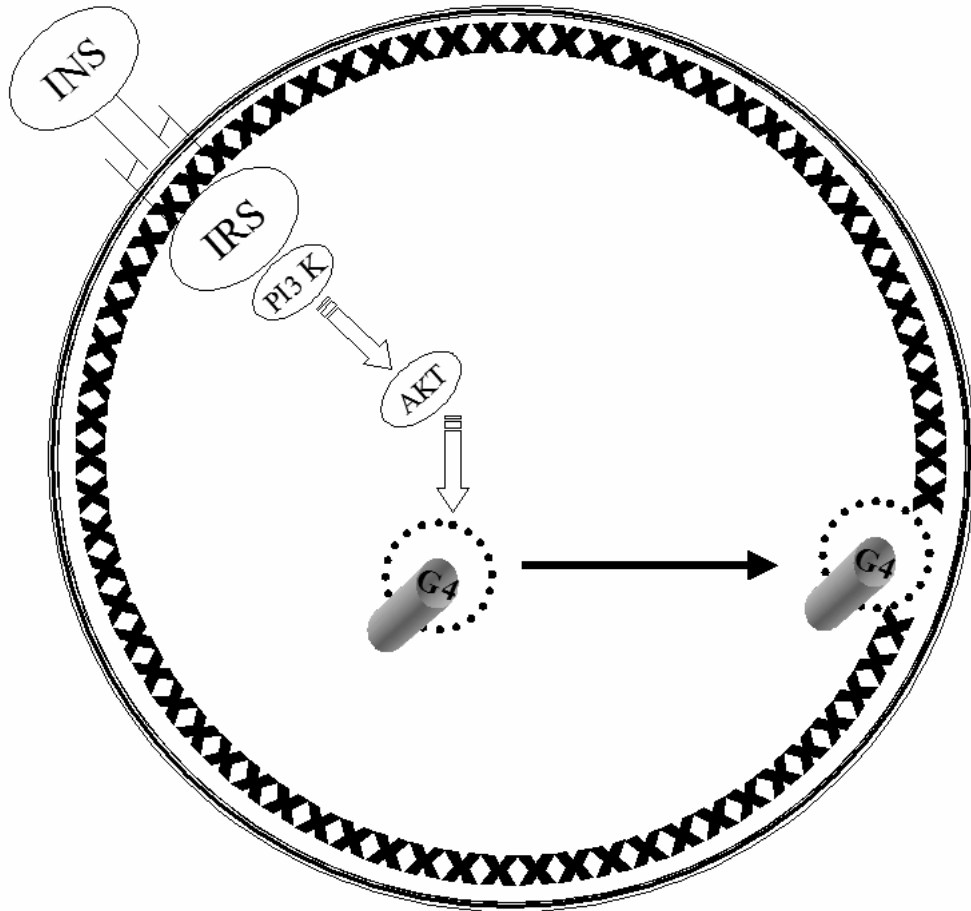
Understanding the insulin signaling pathway is central to understanding NIDDM. This pathway regulates many physiological processes such as glucose homeostasis, gluconeogenesis, glycogenesis and lipogenesis. The insulin signaling pathway is also responsible for the majority of glucose uptake in skeletal muscle and adipose tissue. Impaired glucose uptake causes a host of problems that can lead to the development of NIDDM. Thus, a complete understanding of the cellular and molecular mechanisms involved in the insulin signaling pathway is necessary. Such understandings can lead to medicinal discoveries, which will prevent or treat conditions caused by elevated serum glucose levels.

### **Insulin-Stimulated Glucose Uptake**

Insulin signal transduction results in glucose uptake via the insulin-responsive glucose transporter, GLUT4. GLUT4 vesicles translocate to the plasma membrane as a result of the activation of the insulin-signaling pathway. GLUT4 is responsible for glucose uptake in insulin sensitive tissues such as adipose tissue and skeletal muscle.

When serum glucose levels are high, insulin is secreted from the pancreas into the blood and insulin signaling begins. Insulin binds to its receptor, resulting in a series of events, starting with the activation of the insulin receptor tyrosine kinase via autophosphorylation. The receptor tyrosine kinase then phosphorylates the insulin receptor substrate (IRS), which recruits and activates phosphatidylinositol-3-kinase (PI3

kinase). PI3 kinase activates both the serine/threonine protein kinase (Akt/PKB) and protein kinase C. The activation of these kinases leads to the translocation of GLUT4 vesicles from intracellular pools to the plasma membrane (Fig. 1) (5).



**Figure 1:** Schematic of Insulin-Stimulated Glucose Uptake.

### GLUT4 Vesicle Trafficking and Actin

Previous studies have shown that there is a redistribution of GLUT4 vesicles from a perinuclear region (intracellular pools) to the plasma membrane when adipocytes are stimulated with insulin (6). This translocation results from the interaction of GLUT4

vesicles with the network of actin microfilaments that forms the cytoskeleton (7). In brief, GLUT4 vesicles are translocated to the plasma membrane along actin tracks (8, 9).

Once GLUT4 vesicles translocate to the plasma membrane, GLUT4 vesicles must fuse with the plasma membrane in order for glucose uptake to occur (10). Beneath the plasma membrane, there is a layer of cortical filamentous actin (F-actin) (11). Studies have found that cortical actin must undergo dynamic reorganization for optimal glucose uptake to occur (11). Both disruption and stabilization of the cortical actin layer results in impaired glucose uptake (11). These findings indicate that actin reorganization plays an important regulatory role in insulin-stimulated glucose uptake. In addition, this study also found that the reorganization of cortical actin in adipocytes is the result of insulin stimulation (11). Because cortical actin reorganization is insulin-dependent, it may play an important role in proper insulin-stimulated glucose uptake.

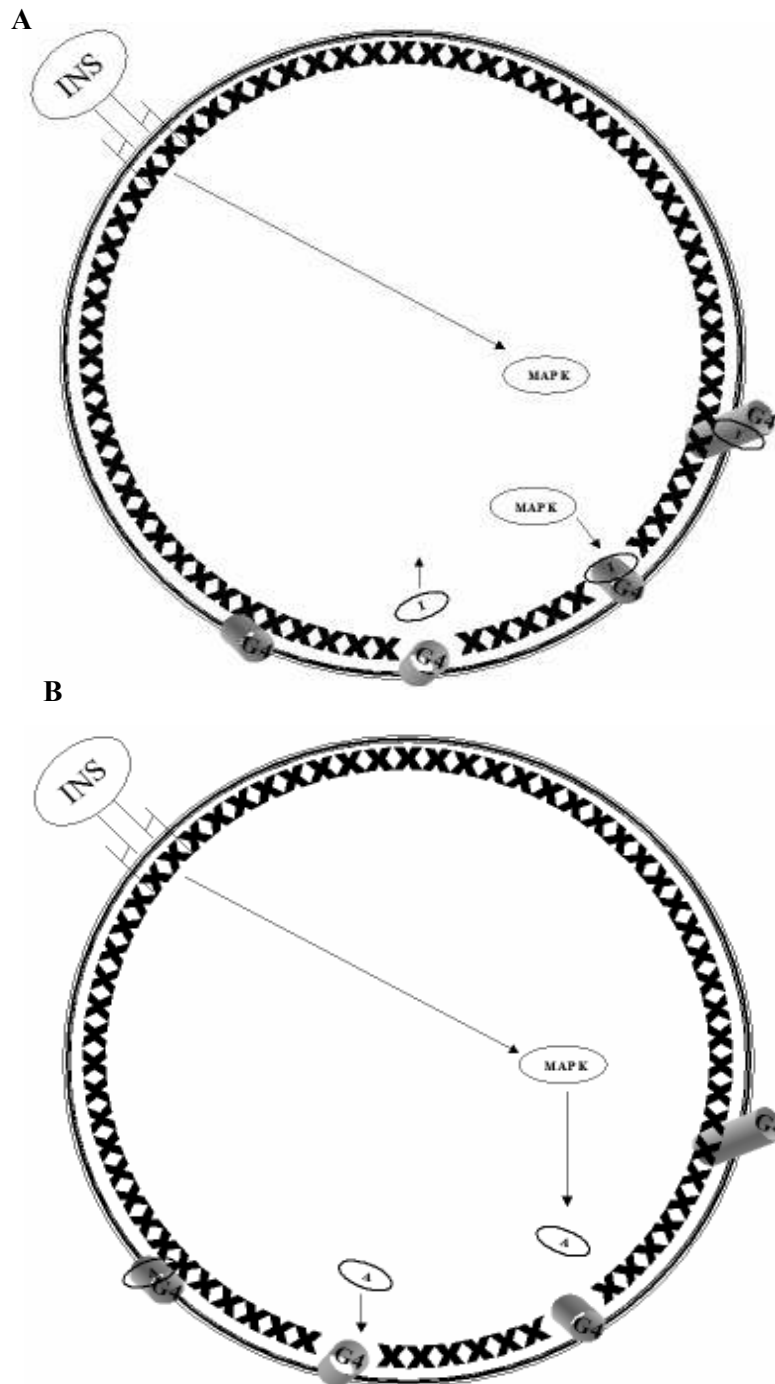
In addition to its role in insulin-stimulated glucose uptake, actin plays a role in vesicle docking and fusion. Studies have described cortical actin as a barrier, which can inhibit vesicle docking and fusion (9). In order for vesicles to fuse to the plasma membrane, there must be disassembly of cortical filamentous actin (32, 33). Once cortical actin is reorganized, vesicles are able to gain access to their proper docking and fusion sites at the plasma membrane (9). This has been demonstrated for GLUT4 vesicles. Previous studies have found cortical actin must be remodeled before GLUT4 vesicles can fuse to the plasma membrane (11). Currently, the mechanism for actin reorganization is still not well understood.

### **GLUT4 Intrinsic Activity**

After fusion, GLUT4 must subsequently be activated before glucose uptake can occur (22). In muscle cells, studies have shown that p38 mitogen-activated protein kinase (p38 MAPK) of the MAPK pathway is necessary for complete GLUT4 activity (12, 22, 23). In addition, p44/p42 mitogen-activated protein kinase (p44/p42 MAPK) was found to increase GLUT4 activity in adipocytes (17). The precise interaction between p38 MAPK or p44/p42 MAPK and GLUT4 is still unknown, but studies have suggested that the increased GLUT4 activity is due to either the interaction of an activator ligand with GLUT4 (Fig. 2B) or the removal of an inhibitory ligand (Fig. 2A) from GLUT4 (12). It is possible that cytoskeletal elements could play a role in the up-regulation of GLUT4 activity by hindering p38 MAPK, p44/p42 MAPK or some other protein from accessing membrane-bound GLUT4.

### **Actin and Myosin**

GLUT4 vesicle trafficking and membrane fusion events require actin reorganization (6, 11, 24, 25). It is uncertain how actin is reorganized during these events, especially during membrane fusion. However, actin reorganization is well characterized in muscle cells. In muscle movement, myosin II is necessary for actin contraction (13). Studies have shown that myosin II plays a necessary role in actin contraction of non-muscle cells for processes such as cell motility, cytokinesis, cellular morphology, vesicle transport and exocytosis (14, 15, 16). Thus, it is possible that myosin II may be responsible for the cortical F-actin reorganization required for GLUT4 vesicle fusion in adipocytes.



**Figure 2: Schematic of the Putative GLUT4 Activation by MAPK.** (A) GLUT4 activation model based on the removal of inhibitory protein. The inhibitory protein is represented as an (I). (B) GLUT4 activation model based on the association of an activator protein. The activator protein is represented as an (A).

Myosin II (conventional myosin) is a hexameric protein consisting of two heavy chains, two essential light chains (ELCs) and two regulatory light chains (RLCs) (15). Myosin II is responsible for the ATP-dependent contraction of actin filaments in skeletal muscle cells and is arranged in an orderly and stable configuration (27). There are several heavy chain isoforms of myosin II. Most vertebrates express two isoforms of myosin II, myosin IIA and myosin IIB. The expression and localization of each isoform are dependant upon cell type (15). The specific functions of each isoform are not yet clear due to their diverse distribution and dynamic localization (15). However, in simple eukaryotes such as *Dictyostelium*, *Acanthamoeba*, and *Saccharomyces*, myosin II has been shown to play an important role in cytokinesis, cell locomotion, and maintenance of cell cortical rigidity (15, 26).

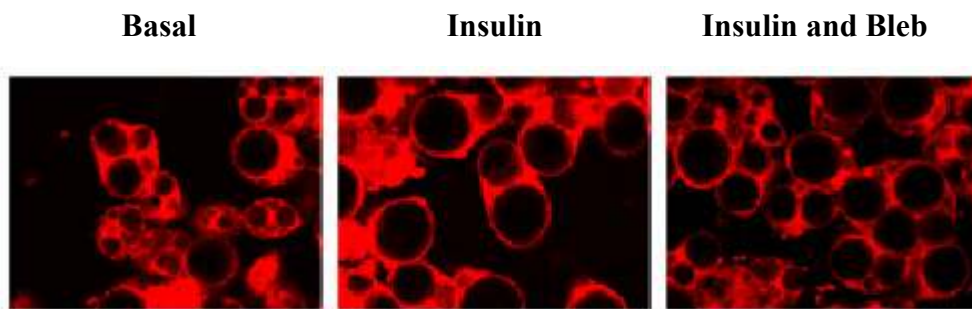
Before myosin II can regulate cellular processes, myosin II must first be activated. Activation of myosin II occurs via phosphorylation of the RLCs of myosin II (15). A kinase responsible for the phosphorylation of the RLCs of myosin II is myosin light chain kinase (MLCK), which activates myosin II (15).

### **Role of Myosin II in Insulin-Stimulated Glucose Uptake**

Recently, our lab has shown that myosin II is involved in insulin-stimulated glucose uptake in adipocytes. Inhibition of myosin II with blebbistatin (a specific inhibitor for non-muscle myosin II) was shown to cause a 66% reduction in insulin-stimulated glucose uptake (8). These results indicate that myosin II is important for insulin-stimulated glucose uptake. We found that myosin II is not necessary for GLUT4 translocation to the plasma membrane using immunofluorescence studies (Fig. 3). In



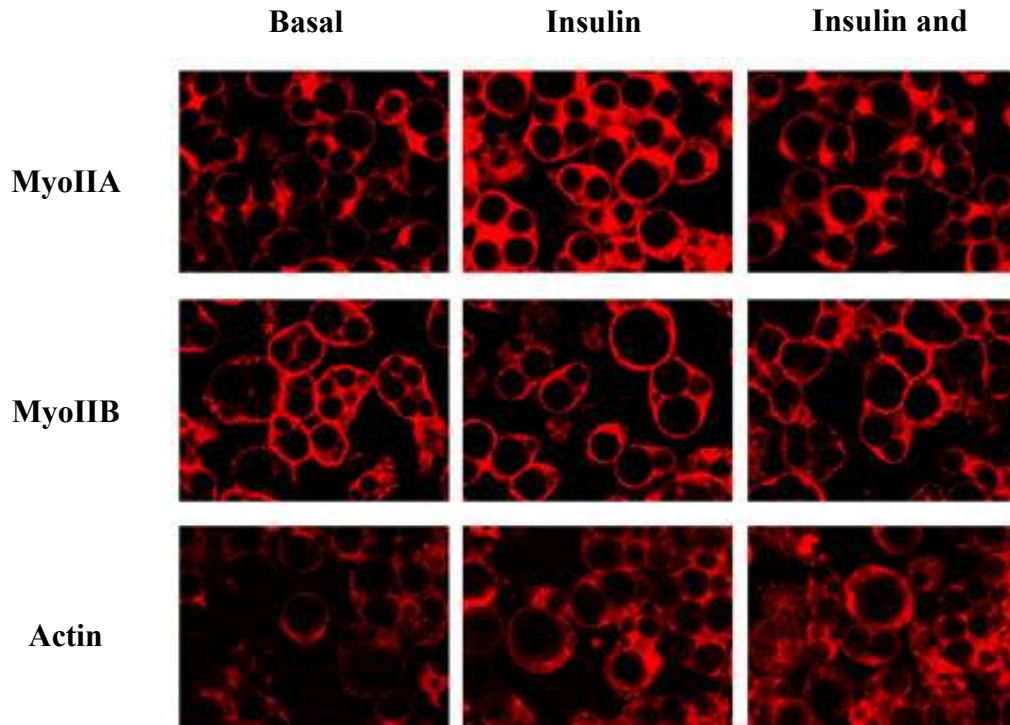
untreated adipocytes, fluorescently labeled GLUT4 vesicles were found to be concentrated in the perinuclear region. Upon insulin-stimulation, GLUT4 vesicles translocated to the plasma membrane. In adipocytes treated with insulin + blebbistatin, GLUT4 vesicles were also shown to translocate to the plasma membrane in a similar manner as that seen in insulin-stimulated adipocytes (8). Taken together, these findings suggest that myosin II is required for GLUT4-mediated glucose uptake but not for GLUT4 vesicle translocation.



**Figure 3: Insulin-stimulated GLUT4 Translocation was not affected by Blebbistatin Treatment.** Adipocytes were treated according to the glucose uptake protocol as described in Materials and methods, and GLUT4 protein was visualized by indirect immunofluorescence using a GLUT4-specific antibody. GLUT4 localization was assayed in whole cells in the basal condition and after stimulation with insulin in the presence and absence of 100 $\mu$ M blebbistatin. The results are representative images from three independent experiments (9).

Our lab has also shown by indirect immunofluorescence that both isoforms of myosin II are expressed in adipocytes (8). In unstimulated adipocytes, myosin IIA was expressed in the perinuclear region of the cells, while myosin IIB was primarily localized at the cell cortex (Fig. 4). However, upon insulin stimulation, myosin IIA translocated to the cell cortex. In contrast, the localization of myosin IIB remained unchanged after

insulin treatment. This suggests that the two isoforms may have different roles in glucose uptake.



**Figure 4: The Effect of Insulin Stimulation on Myosin II Localization in Adipocytes.** 3T3-L1 adipocytes were treated according to the glucose uptake protocol as described in Materials and methods and the myosin II isoforms were visualized by immunofluorescence using either a myosin IIA or myosin IIB-specific antibody. Filamentous actin was visualized using Texas red-phalloidin. Myosin IIA, myosin IIB, and filamentous actin localization was analyzed in the basal condition and after stimulation with insulin in the presence and absence of 100  $\mu$ M blebbistatin using confocal microscopy. The results are representative images from three independent experiments (9).

These findings suggest that myosin II plays a role in insulin-stimulated glucose uptake. Further understanding of myosin II could lead to a possible explanation of how glucose uptake is regulated. This study examined the role of myosin II as it relates to its functional relationship with GLUT4 vesicles. Specifically, the role of myosin II in the

intrinsic activity of GLUT4 was investigated, as well as GLUT4 vesicle fusion and the physical interaction between GLUT4 vesicles and myosin II isoforms. The fundamental hypothesis of this study is that myosin II is involved in the regulation of GLUT4 vesicle fusion and the activity of GLUT4 during insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

## CHAPTER II

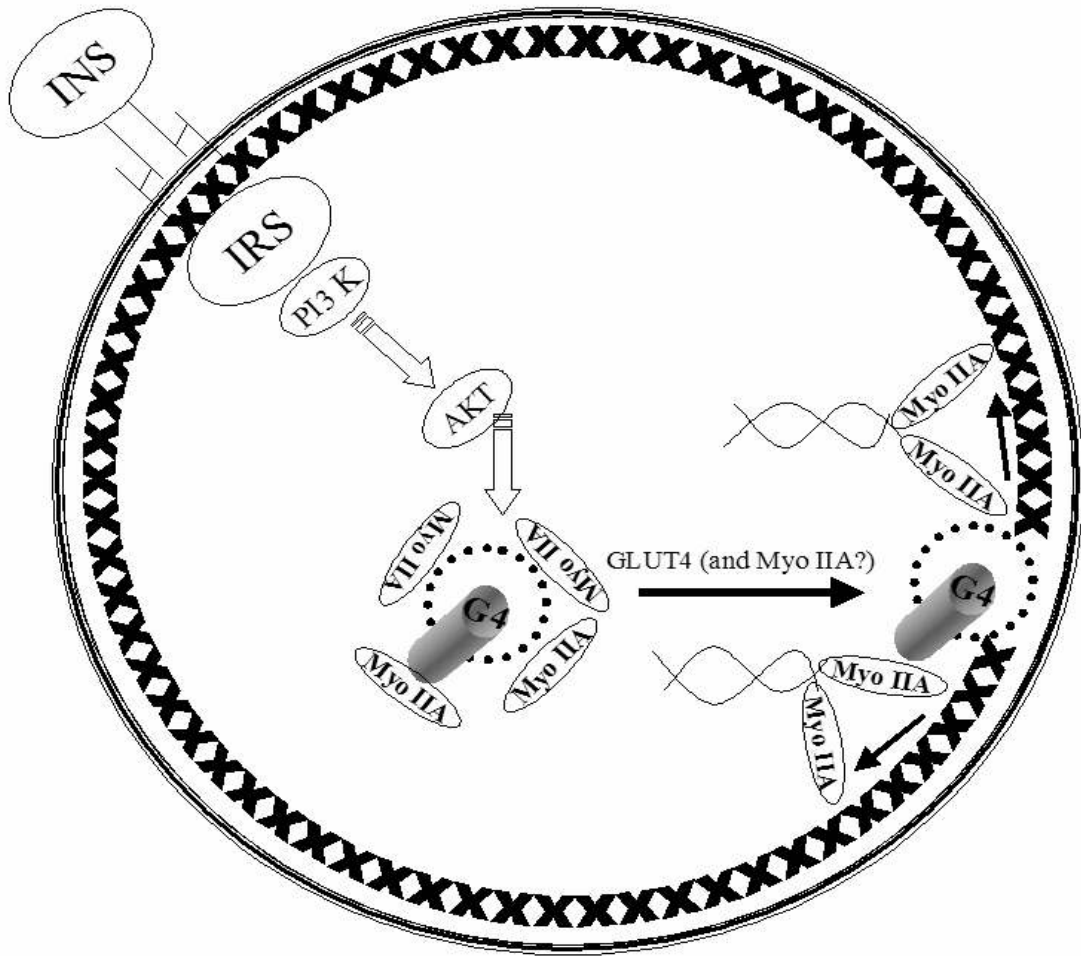
### GOALS AND AIMS

This study encompassed three different aims that examined the functional relationship between myosin II and GLUT4 vesicles during insulin-stimulated glucose uptake.

#### **Aim I: Examination of a Putative Association between Myosin II and GLUT4 Vesicles.**

When adipocytes are stimulated with insulin, GLUT4 vesicles translocate to the plasma membrane (6). In addition, insulin-stimulation leads to cortical actin reorganization, which allows the fusion of GLUT4 vesicles to the plasma membrane (9-11). Although previous studies have found insulin-stimulation is necessary for cortical actin reorganization and GLUT4 vesicle fusion, the mechanism for actin reorganization in membrane fusion events is still unknown.

Actin is known to be reorganized by myosin II in both muscle and non-muscle contexts (13-16). Since myosin II is known to contract (reorganize) actin, it is possible that myosin II is the mechanism by which actin is reorganized during insulin-stimulated glucose uptake, thereby allowing GLUT4 vesicle fusion to the plasma membrane in adipocytes.



**Figure 5:** Schematic of the Putative Physical Relationship between Myosin II and GLUT4 Vesicles in Adipocytes.

Our lab has shown that in the basal state myosin IIA is localized in the perinuclear region of the cell while myosin IIB is localized at the cell cortex. Upon insulin stimulation, myosin IIA relocated to the cell cortex in adipocytes (8). The localization of myosin IIA in the same region of the cell as GLUT4 vesicles suggests a possible association between the two. This association could explain the highly specific coordinated action of actin reorganization at sites of GLUT4 vesicle fusion. If GLUT4

vesicles and myosin IIA translocate to the plasma membrane together, myosin IIA could be responsible for the cortical actin reorganization necessary for vesicle fusion at the plasma membrane. I hypothesized that there is an interaction between myosin IIA and GLUT4 vesicles (Fig. 5). Immunoprecipitation assays were employed to examine a possible myosin II-GLUT4 vesicles interaction.

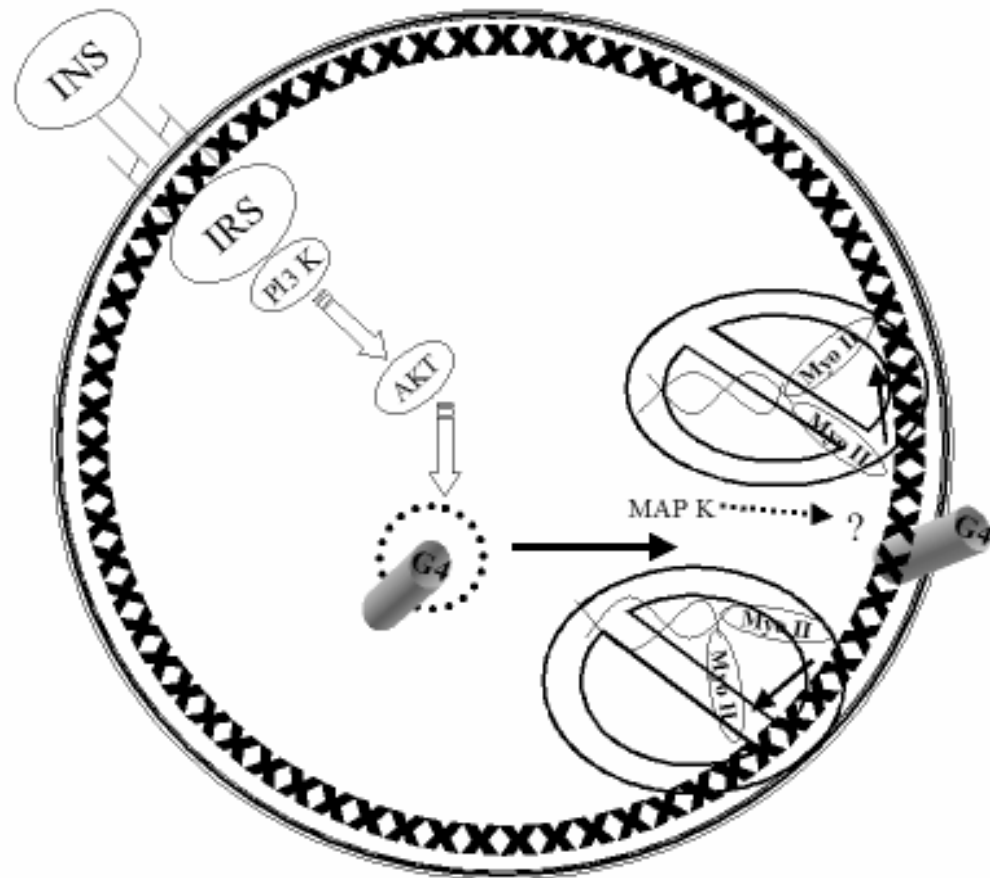
**Aim II: Examination of the Role of Myosin II on the Intrinsic Activity of GLUT4.**

Upon insulin stimulation, GLUT4 vesicles translocate, dock and fuse to the plasma membrane. In order for glucose uptake to occur, however, GLUT4 must also be activated. Studies have suggested GLUT4 activity is up-regulated by its interaction with an activator ligand or by the removal of an inhibitory ligand from GLUT4 (12). GLUT4 activity can also be up-regulated by the MAPK pathway via p38 MAPK or p44/p42 MAPK in muscle cells and adipocytes, respectively (12, 17, 22, 23).

In addition, cortical actin is necessary for GLUT4 translocation and GLUT4-mediated glucose uptake (11). Dynamic cortical actin reorganization is necessary for proper vesicle docking and fusion (9). Conversely, without dynamic reorganization, actin acts as a barrier to prevent vesicle docking and fusion. These observations clearly suggest actin plays a role in GLUT4-mediated glucose uptake. However, the mechanism of actin reorganization is still unclear.

Taken together, these studies suggest a possible mechanism for actin reorganization. The actin cytoskeleton could be acting as a barrier that may hinder p38 MAPK, p44/p42 MAPK or another protein from accessing the membrane-bound GLUT4,

thereby preventing the activation of GLUT4. Thus, actin reorganization may play a role in the up-regulation of GLUT4 activity.



**Figure 6:** Schematic for Putative Role of Myosin II on the Intrinsic Activity of GLUT4.

Actin is reorganized by myosin II-mediated actin contraction (13-16). Myosin II may be reorganizing actin to allow proper GLUT4 activation by a similar mechanism, facilitating GLUT4-mediated glucose uptake. I hypothesized that myosin II is necessary for proper GLUT4 activation (Fig. 6). Insulin-stimulated glucose uptake assays were performed to ascertain if inhibitors of myosin II impair the intrinsic activity of GLUT4.

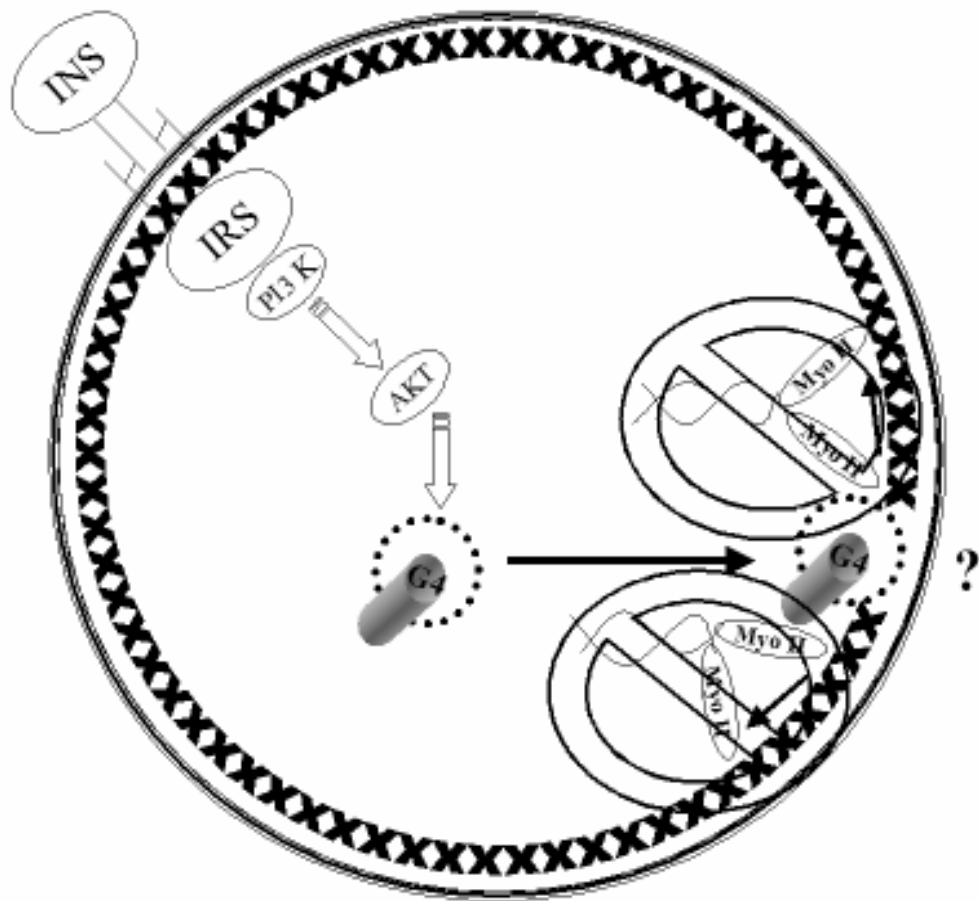
### **Aim III: Examination of the Role of Myosin II in GLUT4 Vesicle Fusion to the Plasma Membrane.**

Previous studies have shown that insulin-stimulation of adipocytes results in the reorganization of cortical actin (11). This reorganization allows GLUT4 vesicles to fuse with the plasma membrane (9, 10). The mechanism of actin reorganization, which allows vesicle fusion, is not well understood. However, studies have found actin to be reorganized by myosin II in both muscle and non-muscle contexts (13, 14, 15, 16). Thus, myosin II may be reorganizing actin to facilitate glucose uptake by allowing the fusion of GLUT4 vesicles to the plasma membrane during insulin-stimulated glucose uptake.

Our lab began to investigate the mechanism of actin reorganization as it relates to insulin-stimulated glucose uptake. Using a specific inhibitor for non-muscle myosin II blebbistatin, we found that inhibition of myosin II lead to inhibition of insulin-stimulated glucose uptake in 3T3-L1 adipocytes, but not GLUT4 translocation to the plasma membrane (8). What is not known is whether myosin II is necessary for GLUT4 vesicle fusion with the plasma membrane.

I hypothesized that myosin II is necessary for GLUT4 vesicle fusion (Fig. 7). Using confocal microscopy, I examined membrane-bound GLUT4 vesicles to determine if the inhibition of myosin II reduced the fusion of GLUT4 vesicles to the plasma membrane. Membrane-bound GLUT4 vesicles were detectable on the cell surface by using a GLUT4 antibody targeting an epitope in the extracellular domain near the N-terminus.





**Figure 7:** The Role of Myosin II and GLUT4 Vesicle Fusion at the Plasma Membrane.

## CHAPTER III

### MATERIALS AND METHODS

#### **Materials**

Dulbecco's modified Eagles's medium (DMEM) for tissue culture was purchased from Gibco (Grand Island, NY). Insulin was obtained from Roche Diagnostics (Indianapolis, IN). Dexamethasone, 3-isobutyl-1-methyl-xanthine, ML-7 (inhibitor of myosin light chain kinase) and myosin IIA antibody were purchased from Sigma (St. Louis, MO). Blebbistatin (a specific inhibitor for non-muscle myosin II) was acquired from Calbiochem (San Diego, CA). Myosin light chain (phospho S20) antibody was purchased from Abcam (Cambridge, MA). GLUT4 antibodies (C-20 and N-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 594 donkey anti-goat IgG and secondary goat anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR).

#### **Methods**

##### Cell Culture

3T3-L1 preadipocytes were cultured in DMEM and 10% calf serum (CS) to confluency. After three days (day 0), preadipocytes were induced to differentiate with the addition of 0.5mM methylisobutylxanthine, 1 $\mu$ M dexamethasone, 1.7 $\mu$ M of insulin,

and 10% fetal bovine serum (FBS) in DMEM. On day 3, media was removed and replaced with media containing 425nM insulin and 10% FBS in DMEM. Cells were then maintained in DMEM containing 10% FBS and media was replaced every two days (18).

#### Insulin Stimulation Assay

Adipocytes were serum starved for 4 hours in DMEM and vehicle (0.1% DMSO) or inhibitor (100 $\mu$ M blebbistatin or 10 $\mu$ M ML-7). Adipocytes were washed two times in Krebs-Ringer Phosphate (KRP) buffer (pH 7.4) containing 128mM NaCl, 4.7mM KCl, 1.65mM CaCl<sub>2</sub>, 2.5mM MgSO<sub>4</sub>, and 5mM Na<sub>2</sub>HPO<sub>4</sub> and then incubated in KRP buffer containing vehicle or various inhibitors as indicated at 37°C. Insulin stimulation was performed at 37°C with 100nM insulin. After a 20-minute incubation period, cells were washed twice in PBS at 4°C. Immediately following, adipocytes were lysed in a buffer containing 25 mM Hepes (pH 7.4), 1% Nonidet P-40, 100 mM NaCl, 2% (v/v) glycerol, 5 mM NaF, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaPPi, and protease and phosphatase inhibitor cocktails. Lysates were gently agitated for 20 minutes at 4°C and centrifuged (14000 g) for 20 minutes at 4°C. Laemmli sample buffer was added to lysates.

#### Immunoprecipitation Assay

3T3-L1 preadipocytes were cultured on 10 cm plates, and induced to differentiate (as above). Adipocytes were subjected to an insulin stimulation assay (as above). After adipocytes were washed with cold PBS washes, they were fixed with 2% formaldehyde for 15 minutes and washed again with cold PBS three times. A hypotonic buffer containing 20mM Tris (pH 7.5), 10mM NaCl, 3mM MgCl<sub>2</sub>, protease inhibitors, and

phosphatase inhibitors were used to lyse the cells. Nonidet P 40 Substitute (NP-40) was added to cells at a concentration of 1:64. The cells were then dounce homogenized (20 strokes). GLUT4 or myosin IIA was immunoprecipitated by using GLUT4 and myosin IIA-specific antibodies, respectively and agarose beads. Immunoprecipitates were centrifuged at 2500g and washed with PBS three times at 4°C. Laemmli sample buffer was added to the immunoprecipitates.

#### Immunoblot Analysis

Immunoprecipates or lysates were subjected to 10% or 15% SDS-PAGE, respectively. The gels were then transferred onto Immobilon-P membranes. To reduce non-specific binding of the antibody, membranes were blocked by soaking in methanol for 10 seconds and dried at room temperature for 20 minutes. After blocking, membranes of immunoprecipates were incubated in GLUT4 or myosin IIA primary antibodies. Membranes were incubated in myosin II RLC or myosin II p-RLC primary antibodies. Membranes were then incubated in secondary antibodies and visualized by enhanced chemiluminescent (ECL) reagents (18).

#### Four Minute Glucose Uptake Assay

3T3-L1 preadipocytes were cultured to confluence and induced to differentiate (see MATERIALS AND METHODS) in 6-well plates. Differentiated adipocytes (day 8-12) were serum-starved for approximately 4 hours in DMEM. After the 4-hour starvation, adipocytes were washed two times in KRP buffer and then incubated in KRP buffer containing vehicle or various inhibitors as indicated at 37°C. Vehicle (0.1% DMSO), 100nM insulin, or 100nM insulin and inhibitor (100µM blebbistatin or 10µM

ML-7) was added to respective well at 20-second intervals. Immediately following, [<sup>14</sup>C]2-deoxy-D-glucose (0.1μCi/well) and 5mM glucose was added to each well at 20-second intervals. Each well was washed twice with phosphate buffered saline (PBS) at 4°C and then lysed with 1mL of a 0.5M NaOH and 0.1% SDS solution. Radio-labeled glucose uptake was quantified as disintegrations per mg protein (18).

#### Immunofluorescence Assay

3T3-L1 preadipocytes were cultured on coverslips, and induced to differentiate (as described previously). Fully differentiated adipocytes were serum starved and stimulated with insulin for 20 minutes (as described previously). Immediately following, adipocytes were fixed with 3.7% formaldehyde and washed with PBS. Adipocytes were then incubated for an hour with a GLUT4 antibody (1:100) targeting an epitope in the extracellular domain near the N-terminus. Cells were washed 5 times in cold PBS for 5 minutes. Then adipocytes were incubated in the secondary antibody, anti-goat Alexaflour 594 for 45 minutes. Cells were washed 5 times in cold PBS for 5 minutes. Finally, coverslips were mounted and viewed using an Olympus IX81 Motorized Inverted Confocal Microscope and FLUOVIEW FV500 software (8, 18).

#### Statistical Analysis

##### **Quantification of Glucose Uptake Assays**

Data are expressed as means ± SEM. The significance of differences between means, set at  $P < 0:05$ , was assessed by Microsoft Excel.

### **Quantification of Immunoblots**

Protein bands were quantified by densitometry using ImageQuant (version 5.2 for Windows) software.

## CHAPTER IV

### RESULTS

#### **Aim I Results: The Association between Myosin IIA and GLUT4 is Insulin Dependent.**

Cortical actin acts as a barrier that inhibits vesicle docking and fusion (9). Studies have found that insulin stimulation leads to cortical actin reorganization, which allows GLUT4 vesicle fusion (9, 11). Previous studies have shown that there is a redistribution of GLUT4 vesicles from a perinuclear region (intracellular pools) to the plasma membrane when adipocytes are stimulated with insulin (6). Also in 3T3-L1 adipocytes, we found that myosin IIA also relocated from a perinuclear region to the plasma membrane upon insulin stimulation (8). The similar redistribution of myosin IIA and GLUT4 vesicles upon insulin stimulation suggests a possible association. This association could explain the highly specific coordinated action of actin reorganization at sites of GLUT4 vesicle fusion. The focus of this study was to determine if myosin IIA and GLUT4 vesicles are associated using immunoprecipitation assays.

Fully-differentiated adipocytes were serum starved in DMEM for 4 hours in the presence of vehicle (0.1% DMSO), vehicle + insulin, insulin + 100 $\mu$ M blebbistatin (a myosin II-specific inhibitor) or insulin + 10 $\mu$ M ML-7 (a myosin light chain kinase inhibitor). The Basal (0.1% DMSO) treatment and Insulin (0.1% DMSO and 100nM insulin) treatment serve as a control for insulin stimulation and for inhibitor effectiveness,

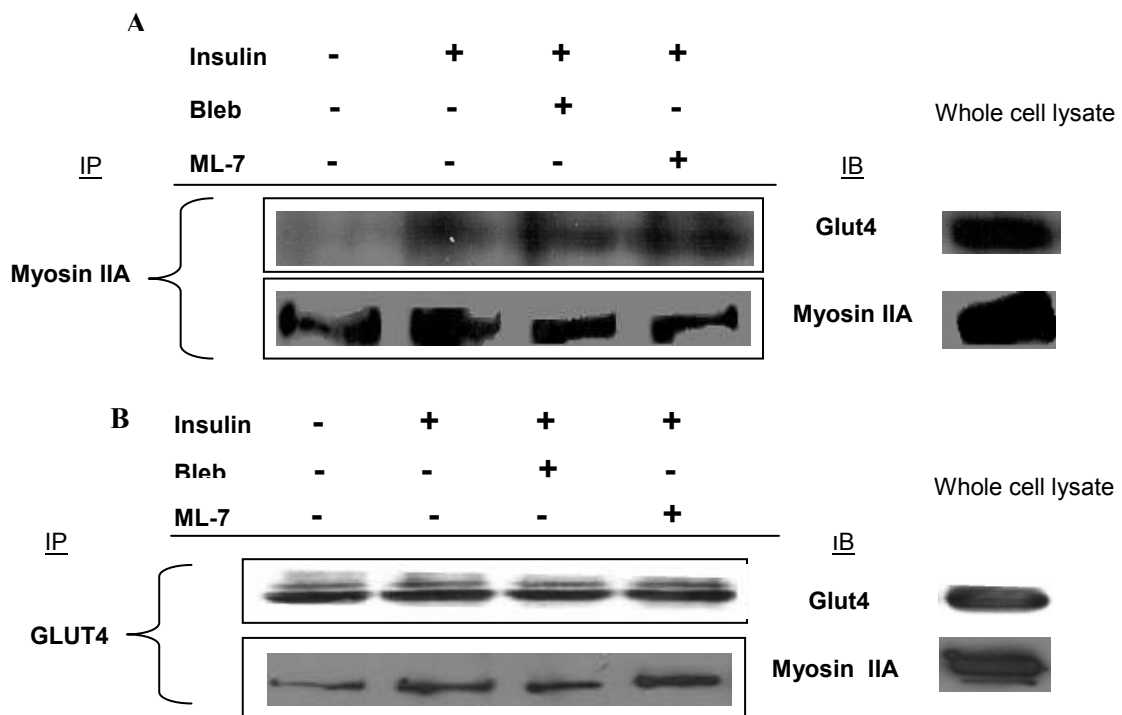
respectively. The Bleb (100nM insulin + 100 $\mu$ M blebbistatin) and ML-7 (100nM insulin + 10 $\mu$ M ML-7) treatments were used to determine the role and mechanism of myosin II in insulin-stimulated glucose uptake. Specifically, the insulin + blebbistatin treatment determined if myosin II has to be associated with actin in order to interact with GLUT4 vesicles interaction. The insulin + ML-7 treatment determined if an association between myosin II and GLUT4 is dependant upon the phosphorylation of myosin II RLC. In addition, the insulin + ML-7 treatment provided insight into the necessary signaling mechanism for this event.

After serum starvation, adipocytes were subjected to insulin stimulation assays and then treated according to the immunoprecipitation protocol (see MATERIALS AND METHODS). Vesicles were isolated by centrifugation. Antibodies for myosin IIA or GLUT4 were used to immunoprecipitate myosin IIA or GLUT4 respectively. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting (Fig. 8A and 8B).

In unstimulated 3T3-L1 adipocytes, low levels of the GLUT4 protein associated with myosin IIA (Fig. 8A). However, upon insulin stimulation, the association between GLUT4 protein and myosin II increased (Fig. 8A). As in insulin stimulated cells, myosin IIA immunoprecipitates from both the insulin + blebbistatin and the insulin + ML-7 treated cells showed a greater association between GLUT4 protein and myosin II than that to unstimulated cells, which resembled the results of the insulin treatment (Fig. 8A). To verify that the increase in GLUT4 was not owing to an increase in myosin IIA, myosin IIA immunoprecipitates were probed with myosin IIA. All samples in all four conditions showed similar levels of myosin IIA protein (Fig. 8A). Thus, any difference



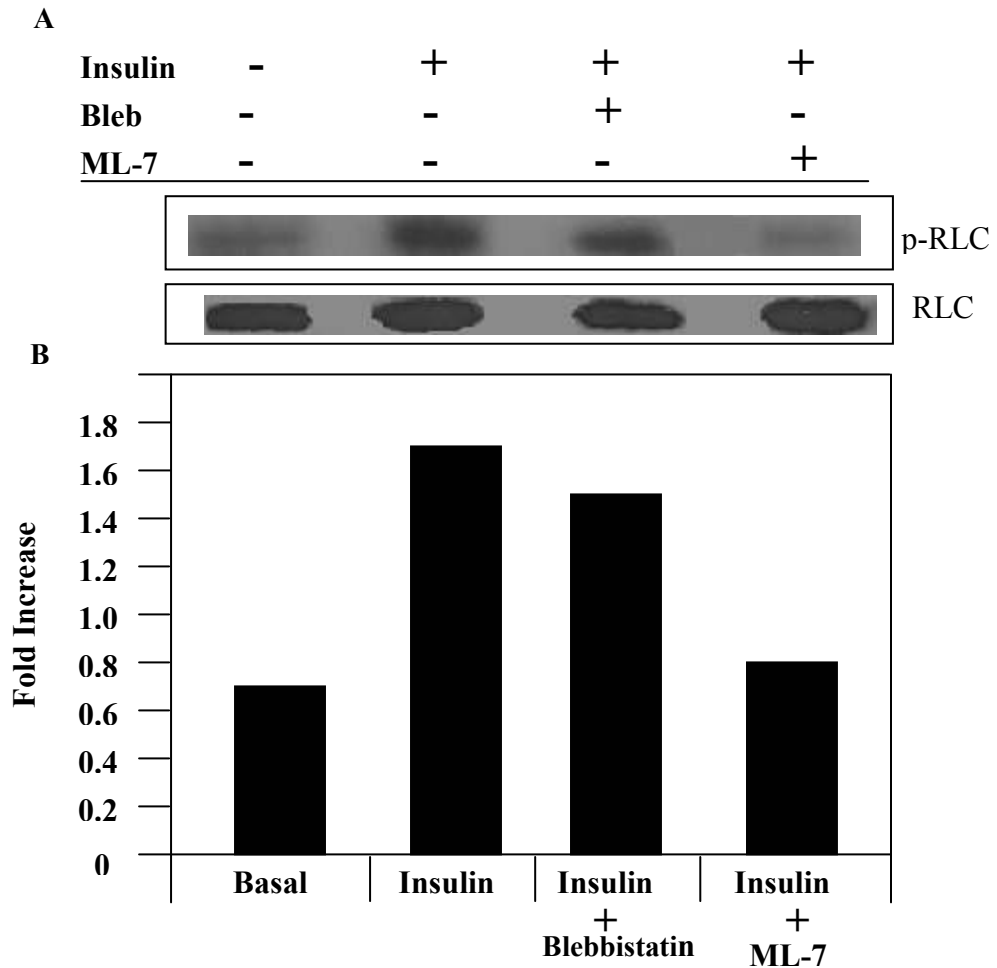
in GLUT4 protein associated with myosin II is not due to increased myosin II, but rather an increase in the amount of GLUT4 associated with myosin II.



**Figure 8: The Association between Myosin IIA and GLUT4 is Insulin Dependent.** 3T3-L1 adipocytes were serum starved for 4 hours and then treated according to the insulin stimulation assay and immunoprecipitation protocol (as described previously in MATERIALS AND METHODS) in unstimulated (Basal), vehicle and insulin (Insulin), insulin and 100  $\mu$ M blebbistatin (Bleb), or insulin and 10 $\mu$ M ML-7 conditions (ML-7). Vesicles were isolated from lysates. Protein was immunoprecipitated with (A) myosin IIA antibody or (B) GLUT4 antibody. All immunoprecipitates were then subjected to 15% SDS-PAGE and immunoblotting. The results are representative of three independent experiments.

The GLUT4 immunoprecipitates verified the results of the myosin IIA immunoprecipitates (Fig. 8B). Unstimulated cells showed low levels of myosin IIA associated with GLUT4 (Fig. 8B). Once stimulated with insulin, the association between myosin IIA and with GLUT4 protein was increased (Fig. 8B). In addition, the insulin + blebbistatin and the insulin + ML-7 treated GLUT4 immunoprecipitates showed a greater

association between myosin IIA and GLUT4 proteins when compared to unstimulated samples (Fig. 8B). Again, the results of both the insulin + blebbistatin and the insulin + ML-7 treated cells were similar to the Insulin treatment.



**Figure 9: Insulin Simulates Phosphorylation of Myosin II Regulatory Light Chain.** 3T3-L1 Adipocytes were serum starved for 4 hours and then treated according to the insulin stimulation assay in unstimulated (Basal), Insulin, Blebbistatin, or ML-7 conditions. (A) Whole cell lysates were prepared and subjected to 10% SDS-PAGE and then immunoblotted using either a phosphorylated myosin II regulatory light chain or myosin II regulatory light chain antibody. (B) The signal was normalized to myosin II regulatory light chain by densitometry. This is representative of three independent experiments.

Previous studies have implicated insulin signaling in increased myosin II regulatory light chain phosphorylation (21). These studies also found that ML-7 was an effective inhibitor of MLCK (21). To verify these findings, additional experiments were performed. Whole cell lysates were subjected to 10% SDS-PAGE and probed with a myosin II p-RLC and a myosin II RLC antibody (Fig. 9A). The signal was then normalized to myosin II regulatory light chain by densitometry (Fig. 9B).

Upon insulin stimulation, the levels of phosphorylated myosin II regulatory light chain increased. The insulin + blebbistatin treatment showed similar levels as that of the insulin treatment. There was, however, a decrease in the levels of phosphorylated myosin II regulatory light chain in the ML-7 (insulin + ML-7) treatment. This result indicates that ML-7 is an effective inhibitor of MLCK and that MLCK phosphorylates the RLC upon insulin stimulation.

#### **Aim II Results: Proper Intrinsic Activity of GLUT4 Requires Myosin II Activity.**

Previous studies have found that myosin II is required for GLUT4-mediated glucose uptake (8). This could reflect a requirement of myosin II for GLUT4 vesicle fusion and/or GLUT4 activity. In order to determine what role myosin II may have on GLUT4 intrinsic activity, fully mature 3T3-L1 adipocytes were serum starved in DMEM for 4 hours in unstimulated (Basal), Insulin, Bleb (insulin + blebbistatin), and ML-7 (insulin + ML-7) treatments.

Inhibitor treatments were used to determine the role and mechanism for myosin II in proper GLUT4 intrinsic activity. The blebbistatin treatment specifically determined if the actin-myosin II association is necessary for proper GLUT4 intrinsic activity, while

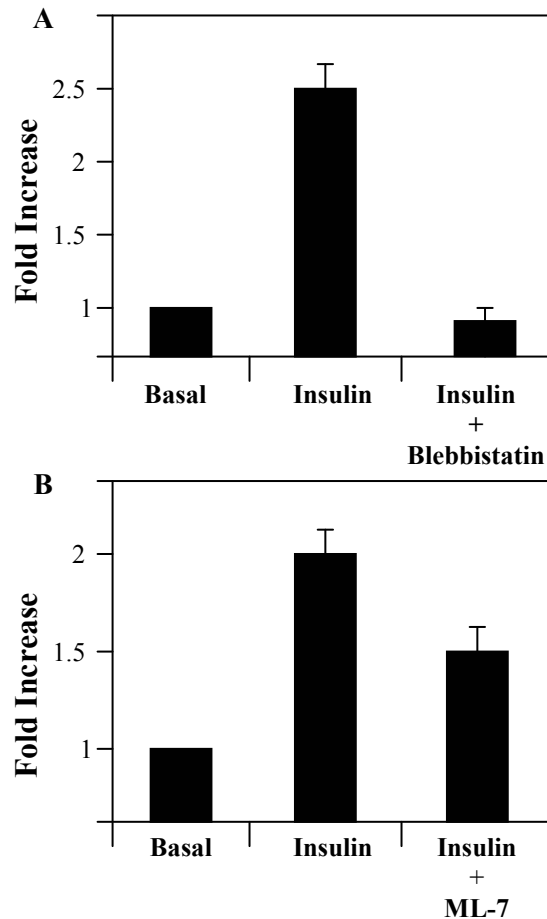
the insulin + ML-7 treatment ascertained the kinase responsible for myosin II activation, which is required for proper GLUT4 intrinsic activity. The insulin + ML-7 treatment also provided insight into the signaling mechanism necessary for proper GLUT4 intrinsic activity.

After serum starvation, adipocytes were subjected to 4-minute glucose uptake assays (see MATERIALS AND METHODS) using [<sup>14</sup>C]2-deoxy-D-glucose. Previous studies have shown that maximum GLUT4 translocation to the plasma membrane occurs between 10-14 minutes (7, 29). Thus, the short 4-minute time period will only examine glucose uptake from embedded GLUT4 vesicles. Our short time frame does not allow enough time for GLUT4 translocation to occur.

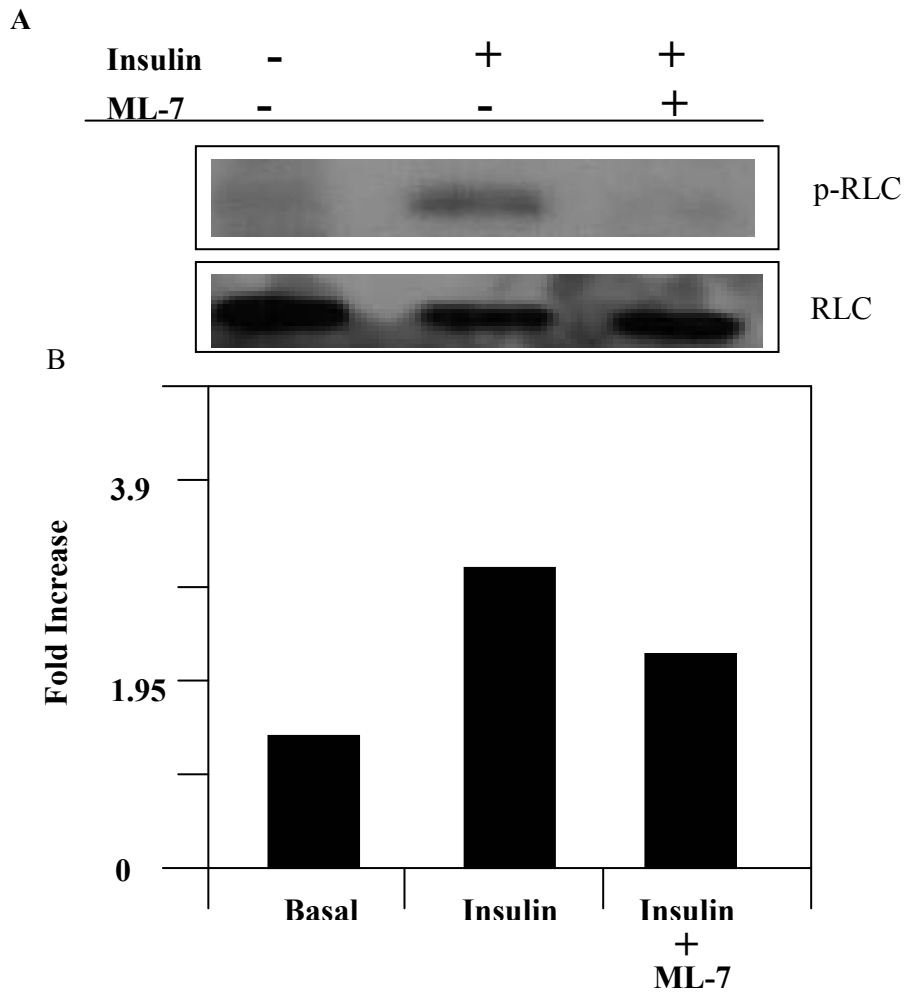
Insulin-stimulated adipocytes showed a two-fold increase in glucose uptake over unstimulated adipocytes (Fig. 10). In contrast, the insulin + blebbistatin treated cells showed inhibition of insulin-stimulated glucose uptake by 68% (Fig. 10A). To determine the signaling mechanism necessary for proper GLUT4 intrinsic activity as related to myosin II, additional assays were performed with the MLCK inhibitor, ML-7. Adipocytes treated with insulin + ML-7 showed an inhibition of glucose uptake by 22% (Fig. 10B) when compared to insulin-stimulated adipocytes.

Additional experiments were performed to examine the phosphorylation of the myosin II regulatory light chain, which is a control for insulin and inhibitor effectiveness. Whole cell lysates were subjected to 10% SDS-PAGE and probed with a phosphorylated myosin II regulatory light chain and a myosin II regulatory light chain antibody (Fig.

11A). Protein was normalized to total myosin II regulatory light chain by densitometry (Fig. 11B).



**Figure 10: Myosin II Increases the Intrinsic Activity of GLUT4 during Insulin-Stimulated Glucose Uptake in 3T3-L1 Adipocytes.** Adipocytes were serum starved in DMEM for 4 hours and then treated according to the glucose uptake protocol (as previously described in MATERIALS AND METHODS) in the Basal, Insulin and Bleb (A) or ML-7 (B) conditions. Adipocytes were then assayed for incorporated [ $^{14}$ C]-deoxy-D-glucose. The data are expressed as a percent of the vehicle control. Results are means  $\pm$  standard error mean (SEM) of three independent experiments.



**Figure 11: Insulin Stimulates Phosphorylation of Myosin II Regulatory Light Chain.** 3T3-L1 Adipocytes were serum starved for 4 hours and then treated according to the insulin stimulation assay (as previously described in MATERIALS AND METHODS) in Basal, Insulin and ML-7 conditions. (A) Whole cell lysates were prepared, subjected to 10% SDS-PAGE and then immunoblotted using either a phosphorylated myosin II regulatory light chain or myosin II regulatory light chain antibody. (B) Immunoblots were quantified by densitometry. Results are representative of three independent experiments.

Immunoblotting analysis showed an increase of phosphorylation of the myosin II RLC upon insulin stimulation. The Bleb (insulin + blebbistatin) treatment showed similar levels of phosphorylated myosin II regulatory light chain as the insulin treatment (data not shown). The ML-7 (insulin + ML-7) treatment showed decreased

phosphorylation of the myosin II RLC (Fig. 11A). These findings indicate that ML-7 is an effective inhibitor of MLCK. In addition, these experiments suggest that phosphorylation of myosin II RLC via MLCK is required for activation of GLUT4 by insulin stimulation.

**Aim III Results: GLUT4 Vesicle Fusion with the Plasma Membrane Requires Myosin II Activity.**

The translocation, docking and fusion of GLUT4 vesicles is what drives insulin-stimulated glucose uptake in mature adipocytes. In several cell types, myosin II has been found to be necessary in vesicle trafficking (16-21). However, recent studies have found myosin II is not required for GLUT4 vesicle translocation, but is necessary for proper GLUT4-mediated glucose uptake (8). What is not known is if myosin II is required for GLUT4 vesicle fusion with the plasma membrane.

To examine if myosin II is required for GLUT4 vesicle fusion, confocal microscopy was used to visualize fusion of GLUT4 vesicles to the plasma membrane of adipocytes. To this end, fully differentiated 3T3-L1 adipocytes were serum starved for 4 hours in unstimulated (Basal), Insulin, Bleb, or ML-7 conditions. The Bleb treatment will ascertain if an actin-myosin II interaction is necessary for GLUT4 vesicle fusion, whereas the ML-7 (insulin + ML-7) treatment will determine how myosin II is activated to aid in GLUT4 vesicle fusion.

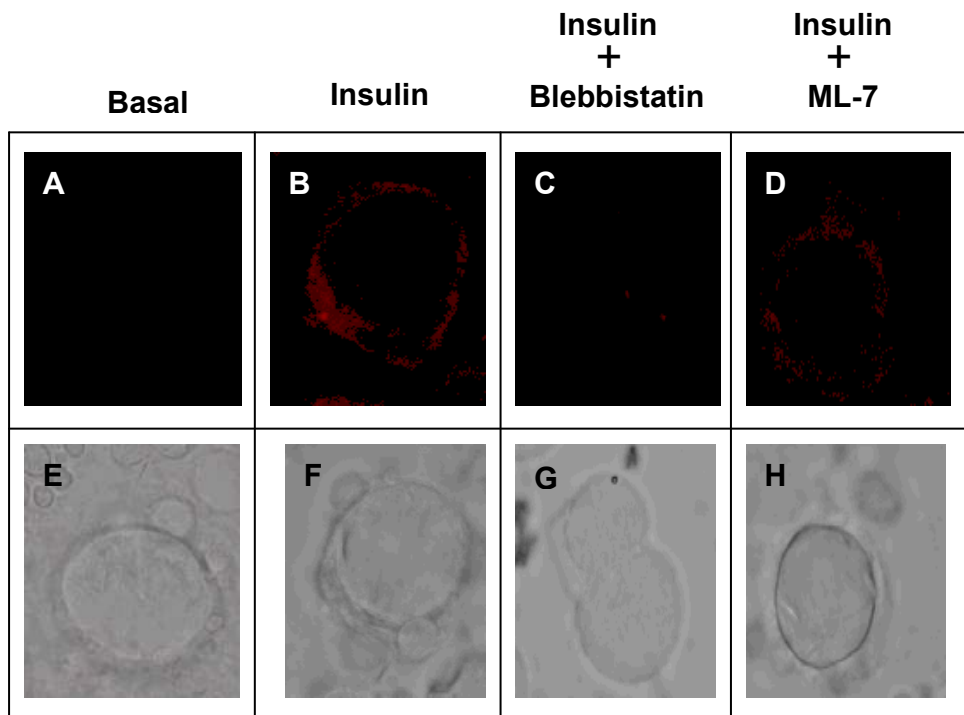
After serum starvation, adipocytes were subjected first to the insulin stimulation assay and then to an immunofluorescence assay (see MATERIALS AND METHODS) using a GLUT4 antibody targeting an epitope in the extracellular domain near the N-

terminus. In standard immunofluorescence assays, cells are permeablized with a detergent, such as Triton-X. However, this study did not permeablize the plasma membrane since only the GLUT4 vesicles properly fused to the plasma membrane were being examined. Properly fused GLUT4 vesicles will have the extracellular domain on the outside of the cell. After adipocytes were probed with the antibody, they were then visualized by confocal microscopy. Differential interference contrast (DIC) images of each treatment (Fig. 12 – panels E-H) were taken in addition to the confocal images to verify the presence of adipocytes.

As expected, insulin-stimulated adipocytes showed a dramatic increase in fused GLUT4 vesicles when compared to unstimulated adipocytes (Fig. 12 – compare panel B to A). This confirms previous studies, which found that GLUT4 vesicles relocate to the plasma membrane upon insulin stimulation (6). To determine if the myosin II- mediated cortical actin contraction is necessary for the fusion of GLUT4 vesicles to the plasma membrane, adipocytes were treated with insulin + blebbistatin. The adipocytes treated with insulin + blebbistatin showed decreased levels of fused GLUT4 vesicles when compared to insulin-stimulated cells (Fig. 12 – compare panel C to B). The insulin + ML-7 treatment was performed to provide insight into the signal mechanism required for myosin II activation during cortical actin contraction. Adipocytes treated with insulin + ML-7 showed a substantial decrease in the level of properly fused GLUT4 vesicles when compared to adipocytes stimulated with insulin alone (Fig. 12 – compare panel D to B). However, the insulin + ML-7 treated adipocytes failed to inhibit GLUT4 vesicle fusion to



the same extent as adipocytes treated with insulin + blebbistatin (Fig. 12 – compare panels D to C).



**Figure 12: Inhibition of Myosin II Activity Inhibits GLUT4 Vesicle Fusion with the Plasma Membrane.** Fully-matured 3T3-L1 adipocytes were serum starved in DMEM for 4 hours and then treated according to the immunofluorescence protocol (as described in MATERIALS AND METHODS). Fused GLUT4 vesicles were visualized using a GLUT4-specific antibody targeting an epitope in the extracellular domain near the N-terminus. GLUT4 localization in adipocytes was examined in (A) the basal condition and (B) after stimulation with insulin (C) in the presence of 100 $\mu$ M blebbistatin or (D) 10 $\mu$ M ML-7. Differential Interference Contrast (DIC) images of each treatment were taken as well (E-H). The results are representative images from three independent experiments.

CHAPTER V  
DISCUSSION AND CONCLUSIONS

With the ever-increasing prevalence of type II diabetes worldwide, scientists are seeking to gain a comprehensive understanding of insulin-stimulated glucose uptake. The purpose of this study was to further examine a novel finding implicating myosin II in facilitating glucose uptake. Specifically, this study examined the role of myosin II in GLUT4 activity and GLUT4 vesicle fusion.

**Aim I: Examination of a Putative Association between Myosin II and GLUT4 Vesicles.**

Summary Review

In insulin-stimulated glucose uptake, GLUT4 vesicles will translocate from a perinuclear region to the plasma membrane of insulin sensitive tissues such as adipose tissue. The redistribution of GLUT4 vesicles upon insulin stimulation is the result of interactions between GLUT4 vesicles and actin microfilaments (7). When GLUT4 vesicles translocate to the membrane, they then must fuse with the plasma membrane.

Previous studies have shown that there is a layer of cortical actin beneath the plasma membrane in adipocytes and that the cortical actin layer must be reorganized to allow vesicles to fuse with the plasma membrane (9, 11). The mechanism of cortical actin remodeling is not well understood. However, previous studies have found actin to be reorganized by myosin II (conventional myosin) in both muscle and non-muscle cell

contexts (13 – 16). In addition, studies found that myosin II-mediated cortical actin contraction is necessary for vesicle fusion in chromaffin cells (16).

Previous studies have found that cortical actin will reorganize upon insulin stimulation, which is necessary for glucose uptake and/or vesicle fusion (9, 11). Because of these findings, our lab sought to examine the mechanism of cortical actin reorganization in insulin-stimulated glucose uptake (9-11). In addition, other studies have implicated myosin II in actin reorganization (13-16). Based upon myosin II implication in actin reorganization, we examined myosin II to determine if it had a role in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Our lab found that myosin II plays a role in GLUT4-mediated glucose uptake.

Discussion of findings: The association between myosin IIA and GLUT4 is insulin dependent

In previous studies, our lab found myosin IIA redistribution to mimic GLUT4 vesicle localization and translocation upon insulin stimulation. What was not known is whether GLUT4 vesicles and myosin IIA travel together to the plasma membrane. Through immunoprecipitation assays, we determined that an association exists between GLUT4 vesicles and myosin IIA to allow simultaneous movement. Our results indicated that GLUT4 vesicles and myosin IIA associate upon insulin-stimulation. There was a greater association between GLUT4 and myosin IIA in insulin-stimulated cells when compared to the unstimulated cells. This, however, did not provide insight into how myosin II is associating with GLUT4 vesicles.

Myosin II is known to contract actin. Thus, myosin IIA may associate with GLUT4 vesicles through an interaction with actin. Previous studies have observed actin on transport vesicles, such as GLUT4 vesicles (28). To determine how myosin IIA associates with GLUT4 vesicles, two inhibitors (blebbistatin and ML-7) were used in these immunoprecipitation assays.

Blebbistatin is a myosin II-specific inhibitor that impairs the physical interaction between myosin II and actin. We found that blebbistatin did not significantly alter the level of myosin IIA associated with GLUT4 vesicles upon insulin stimulation. Thus, there was a greater association between GLUT4 and myosin IIA in the insulin + blebbistatin treated cells than in unstimulated cells. Taken together, our findings suggest that the association between myosin IIA and GLUT4 vesicles is not mediated by actin.

ML-7 is an inhibitor of MLCK. MLCK is a kinase that activates myosin II by phosphorylating the regulatory light chain of myosin II. In the immunoprecipitation assays, the insulin + ML-7 treatment provided insight into the necessary signaling mechanism for myosin IIA activation. We found that ML-7 did not significantly alter the level of myosin IIA associated with GLUT4 vesicles upon insulin stimulation. The insulin + ML-7 treated cells showed a greater association between GLUT4 and myosin II than that of unstimulated cells. Thus, these results suggest that the insulin-stimulated association of myosin II with GLUT4 does not require myosin II activation by MLCK.

In conclusion, the immunoprecipitation assays found that an association between GLUT4 vesicles and myosin IIA occurs upon insulin stimulation. However, this association is not due to myosin II-actin interactions or the activation of myosin II by

MLCK. We are the first to show an association between myosin IIA and GLUT4 vesicles upon insulin-stimulation. Although novel, our findings are consistent with other studies that determined myosin IIA can bind to vesicles (30, 31).

Also, the results of this study determined that the myosin IIA-GLUT4 vesicle association is not dependent upon myosin IIA interaction with actin. These results must be further verified. Previous studies have observed actin on GLUT4 vesicles (28). Logically, myosin IIA should be interacting with this actin on GLUT4 vesicles. However, upon verification of these results, this study suggests that myosin II may be able to interact with other proteins other than actin. This finding would be consistent with other studies that have shown myosin II to interact with other proteins, such as p200, a peripheral membrane protein (31).

Our study also found that the insulin dependent myosin IIA-GLUT4 vesicle association is not reliant upon myosin IIA activation by MLCK. However, myosin II may still require activation. Additional immunoprecipitation assays should be performed to determine if other kinases are responsible for myosin IIA activation during this association event.

#### Future studies

The results of this study suggest myosin IIA is associated with GLUT4 vesicles via another protein besides actin. To provide further confirmation and credence to these findings, the next series of experiments performed should verify the results of this study. One suggestion is to perform immunoprecipitation assays with isolated GLUT4 vesicles using an actin antibody. Immunoprecipitates will then subjected to SDS-PAGE and

immunoblot analysis using actin, myosin IIA, and GLUT4 antibodies. The results will further verify myosin IIA is associated with GLUT4 vesicles through another protein. Additional future studies should ascertain the identity of this protein.

## **Aim II: Examination of the Role of Myosin II on the Intrinsic Activity of GLUT4.**

### Summary Review

Studies have found that dynamic actin reorganization is necessary for proper GLUT4-mediated glucose uptake (11). Cortical actin must be reorganized to allow vesicle fusion (9). However, cortical actin could be acting as a barrier to other proteins such as those necessary to activate GLUT4 (9). Previous studies have found GLUT4 vesicles will translocate, dock and fuse to the plasma membrane upon insulin stimulation (7-10). After GLUT4 vesicles fuse to the plasma membrane, GLUT4 must be activated in order for glucose uptake to occur (22). The exact mechanism for GLUT4 activation is still unknown, but studies have suggested that GLUT4 activity is up-regulated by either the interaction of an activator ligand or the removal of an inhibitory ligand (12). In addition, previous studies have found that two kinases, p38 MAPK and p44/p42 MAPK stimulated GLUT4 activity in skeletal muscle cells and adipocytes, respectively (12, 17, 22, 23). Because the actin cytoskeleton may be hindering proteins such as p38 MAPK and p44/p42 MAPK from proper access to embedded GLUT4, it is important to determine whether cortical actin reorganization facilitates GLUT4 activation.

### Discussion of findings: Intrinsic activity of GLUT4 requires myosin II activity

As with the previous aim, this aim examined the role myosin II may play in cortical actin reorganization in insulin-stimulated glucose uptake. Specifically, we

determined if myosin II plays a role in the intrinsic activity of GLUT4. To ascertain the role myosin II may play in the intrinsic activity of GLUT4 to transport glucose, we performed 4-minute insulin-stimulated glucose uptake assays using [<sup>14</sup>C]2-deoxy-D-glucose. The 4-minute time frame was utilized in order to examine only the membrane-embedded GLUT4. Previous studies have found maximum GLUT4 translocation to occur between 10-14 minutes (7, 29). By performing glucose uptake experiments during this short time frame, we are able to eliminate glucose uptake due to GLUT4 translocated from intracellular pools.

The focus of this study was to examine the possible role of myosin II on the intrinsic activity of GLUT4 during insulin-stimulated glucose uptake. To ascertain the role of myosin II, two inhibitors (blebbistatin and ML-7) were used in this assay.

We found that blebbistatin inhibited glucose uptake by 68% when compared to insulin-stimulated cells. This indicates that myosin II-mediated actin contraction is necessary for the optimal intrinsic activity of previously embedded GLUT4 vesicles to transfer glucose into adipocytes. Thus, myosin II activity plays a role in cortical actin reorganization by possibly allowing essential proteins to interact with GLUT4, which will simulate GLUT4 activity.

We found that ML-7 inhibited glucose uptake by 22% when compared to insulin-stimulated cells. This result provides insight into the necessary signaling mechanism for the activation of myosin II. MLCK is necessary for the activation of myosin II during insulin-stimulated glucose uptake.

When compared to the results of the blebbistatin treatment, the ML-7 (insulin + ML-7) treatment results did not provide a comparable inhibitory effect. There are a variety of explanations for this effect. One explanation may be due to other kinases. Since MLCK activity is inhibited, other kinases may be compensating for MLCK by phosphorylating the RLC. Another explanation for ML-7 smaller inhibitory effect could involve the time period of myosin II activation. Myosin II may have been activated prior to the end of the 4-minute time period. Regardless, this inhibition indicates that MLCK is a necessary signaling molecule to activate myosin II, which allows optimal GLUT4-mediated glucose uptake.

The results of this study supported previous findings. We verified that glucose uptake is increased in insulin-stimulated cells when compared to unstimulated cells during the short 4-minute time period. This confirms that insulin-stimulation causes an increase in the intrinsic activity of embedded GLUT4. Under normal physiological conditions, low levels of GLUT4 are always present at the plasma membrane regardless of an insulin signal. The amount of GLUT4 is determined by the insulin signaling. If serum glucose levels are normal, additional insulin will not be secreted and most GLUT4 vesicles will be sequestered in the perinuclear region of the cell. However, it is important to remember that GLUT4 is still present at the plasma membrane. Since, GLUT4 must be activated after it fuses with the plasma membrane, there can actually be fused GLUT4 vesicles that are not actively transporting glucose into adipose or skeletal muscle tissue.

In conclusion, myosin II-mediated cortical actin reorganization is necessary for the intrinsic activity of GLUT4 vesicles to allow insulin-stimulated glucose uptake in



3T3-L1 adipocytes. Our study is the first to find that myosin II is necessary for the upregulation of GLUT4 activity.

This study also found that myosin II activation is a result of its phosphorylation by MLCK. Thus, myosin II requires activation by MLCK for full upregulation of GLUT4 intrinsic activity. Our findings are the first to provide insight into the necessary signaling mechanism of GLUT4 activation via of myosin II activation. A complete signaling pathway for the activation of myosin II to allow GLUT4 activation will provide new understanding to impaired insulin sensitivity.

#### Future studies

While cell lines such as 3T3-L1 adipocytes are very useful, they may not function exactly like a living organism. Thus, the next set of experiments should be an *in vivo* study using cells from myosin IIA knockout mice. *In vivo* glucose uptake and insulin stimulation assays can provide insight into the role myosin II plays during insulin-stimulated glucose uptake in a living model.

#### **Aim III: Examination of the Role of Myosin II in GLUT4 Vesicle Fusion to the Plasma Membrane.**

#### Summary Review

Previous studies have determined adipocytes contain a F-actin layer beneath the plasma membrane in adipocytes, which must be reorganized to allow vesicle fusion with the plasma membrane (9, 11). However, this mechanism of cortical actin reorganization is not well understood, but studies have found actin to be reorganized by myosin II in both muscle and non-muscle cell contexts (13 – 16). In addition, our lab found that

myosin II was necessary for proper insulin-stimulated glucose uptake, but not required for GLUT4 vesicle translocation to the plasma membrane (8). We also found that the inhibition of myosin II did not affect the phosphorylation of Akt, which is a distal event in the insulin signaling pathway. These findings suggest that myosin II is involved in the docking and/or fusion of GLUT4 vesicles to the plasma membrane.

Discussion of findings: GLUT4 vesicle fusion with the plasma membrane requires myosin II activity

The last aim of this study was to determine if myosin II activity was necessary for the fusion of GLUT4 vesicles to the plasma membrane. Confocal microscopy was used to examine the role of myosin II in proper GLUT4 vesicle fusion. To ensure that only fused GLUT4 vesicles were being examined, no Triton-X was used to prepare cells for the confocal study. Triton-X is a detergent that permeabilizes cells to allow antibodies access to intracellular proteins. The antibody employed in this study specifically targets the extracellular domain near the N-terminus of GLUT4. The extracellular domain of GLUT4 will be on the outside of the cell if GLUT4 vesicles have properly fused with the plasma membrane.

In all physiological states, there are always some GLUT4 vesicles embedded in the membrane, but most are sequestered in intracellular pools within the cell when they are not required to transfer glucose. Insulin-stimulation of adipocytes will cause perinuclear GLUT4 vesicles to translocate, dock, and fuse to the plasma membrane. The results of the insulin treatment confirmed this fact. Our study verified that the concentration of fused GLUT4 vesicles will increase upon insulin-stimulation (6, 7, 29).

The last aim of this study was to determine if myosin II activity was necessary for the fusion of GLUT4 vesicles to the plasma membrane. To determine the role of myosin II, two inhibitors (blebbistatin and ML-7) were used.

The blebbistatin treatment was used to establish if the physical interaction between myosin II and actin was necessary to allow the fusion of GLUT4 vesicles. The results showed GLUT4 vesicle fusion was impaired when myosin II was inhibited by the blebbistatin treatment. This finding suggests that myosin II is involved in the mechanism necessary to reorganize cortical actin to allow GLUT4 vesicle fusion.

The ML-7 (insulin + ML-7) treatment was used to determine how myosin II must be activated in order to interact with cortical actin. The results of this experiment found a significant reduction in GLUT4 vesicle fusion to the plasma membrane when MLCK was inhibited. This finding indicates that MLCK plays a key role in myosin II activation, and thus GLUT4 vesicle fusion with the plasma membrane.

In conclusion, our study indicates that myosin II activity is necessary for proper GLUT4 vesicle fusion to the plasma membrane. Our findings are the first to describe a role for myosin II activity in GLUT4 vesicle fusion. This study has provided insight into a necessary structural event for GLUT4 vesicle fusion. Some studies have suggested that myosin II might play a necessary role in vesicle fusion (16), but our findings are the first to actually demonstrate that myosin II plays a role in GLUT4 vesicle fusion. In addition, this study suggests that GLUT4 vesicle fusion requires the activation of myosin II by MLCK. Our findings are the first to provide insight into the necessary signaling mechanism of myosin II activation for GLUT4 vesicle fusion. Additional studies can

now be performed to ascertain upstream signaling events for the activation of myosin II. Characterization of the pathway for GLUT4 vesicle fusion via myosin II activation will provide additional insight into impaired insulin sensitivity.

### Future Studies

Previous studies performed in our lab found that myosin IIA will relocate to the plasma membrane upon insulin-stimulation. Thus, we believe myosin IIA is the isoform responsible for GLUT4 vesicle fusion. However, our study also found that myosin IIB is localized at the plasma membrane in unstimulated and stimulated conditions. The next series of studies should elucidate the roles of each isoform in GLUT4 vesicle fusion. This study will examine if one isoform is responsible for GLUT4 vesicle fusion or if both myosin II isoforms play a role in GLUT4 vesicle fusion. To ascertain this information, confocal studies will be performed in *in vitro* (3T3-L1 adipocyte cell line) and cells from myosin IIA or myosin IIB knockout mice.

### **Conclusions**

Insulin-stimulated glucose uptake is a highly regulated physiological process. Previous studies have found that dynamic cortical actin reorganization is necessary for optimal GLUT4 vesicle fusion as well as GLUT4-mediated glucose uptake. Until recently, the dynamic remodeling of cortical actin was not well understood. In this and previous studies, our lab has examined the role of myosin II during insulin-stimulated glucose uptake. Our findings implicate myosin II as the mechanical mechanism responsible for cortical actin reorganization.

This study found that myosin IIA associates with GLUT4 vesicles upon insulin stimulation. However, this association is not due to the myosin II-actin interactions or the activation of myosin II by MLCK. Although, the activation of myosin II may result from other kinases, further studies possibly using confocal microscopy should be performed to verify MLCK is responsible for myosin II activation. For example, myosin IIA and GLUT4 proteins can be observed at different time points to determine exactly when myosin II associates with GLUT4 vesicles.

This study also found that myosin II-mediated cortical actin contraction is necessary for proper GLUT4 vesicle fusion as well as the intrinsic activity of GLUT4. In both instances, MLCK activated myosin II via phosphorylation of myosin II RLC.

In addition, our study is the first to demonstrate the dual role that myosin II plays in GLUT4-mediated glucose uptake. Our study suggests that the myosin II-mediated actin contraction is necessary to allow GLUT4 vesicles to fuse with the plasma membrane. We also implicate myosin II-mediated actin contraction as a terminal insulin signaling event for GLUT4 activation.

Taken together, our findings indicate the necessary role myosin II has on insulin-stimulated glucose uptake. Any impairment of myosin II activity or activation can cause detrimental effects during GLUT4-mediated glucose uptake. Continued research is needed to further examine the role and activation of myosin II in GLUT4-mediated glucose uptake. A thorough understanding of these processes can lead to practical innovations to treat individuals suffering from decreased insulin sensitivity and glucose uptake.

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