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Insulin-stimulated glucose uptake requires the activation of the nonmuscle motor protein myosin II. Our previous studies using pharmacological inhibitors suggest that insulin signaling results in the phosphorylation of myosin IIA during insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Since pharmacological inhibitors are not specific, we wanted to use a siRNA approach to complement our previous studies. In this report we demonstrate that knockdown of myosin IIA using a myosin IIA-specific siRNA resulted in impaired insulin-stimulated glucose uptake. To delineate the signaling pathway involved we asked if siRNA specific to myosin light chain kinase (MLCK), an upstream regulator of myosin IIA, would have the same effect. While we did not observe a reduction in MLCK or impairment of insulin-stimulated glucose uptake, we were able to observe that MLCK is phosphorylated upon insulin stimulation suggesting a role for MLCK in insulin-stimulated glucose uptake. Next, we used siRNA specific to extracellular-signal regulated kinase 2 (ERK2) to establish a role for this kinase in insulin-stimulated glucose uptake. Our results revealed that knockdown of ERK2 resulted in reduced phosphorylation of MLCK. Knockdown of ERK2 also impaired insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Lastly, we used siRNA specific to the calcium/calmodulin kinase II delta isoform to explore its role as a potential upstream activator of ERK2. Only a slight decrease in CaMKIIδ expression was achieved, but this did not result in a significant change in insulin-stimulated glucose uptake. Taken together, our results suggest that myosin IIA is involved in insulin-

stimulated glucose uptake and that it is regulated via MLCK phosphorylation by ERK2 resulting in regulation of glucose uptake upon insulin stimulation in 3T3-L1 adipocytes.

CHARACTERIZATION OF THE ROLE OF MYOSIN II DURING INSULIN-STIMULATED GLUCOSE UPTAKE IN 3T3-L1 ADIPOCYTES

by

Shelly Woody

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Approved by

_____

Committee Chair

Dedicated to my loving and supportive husband, Dean, and our two little girls, India and our soon-to-be-born baby girl, Nyree.

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

Diabetes mellitus is a metabolic disorder that is characterized by a malfunction in glucose homeostasis causing blood glucose levels to be dangerously elevated. According to the International Diabetes Foundation, approximately 246 million people worldwide were diagnosed with diabetes in 2006 with ninety-five percent of those individuals having type II diabetes. Type II diabetes is characterized by insulin resistance and obesity. Insulin resistance results when chronic high levels of glucose causes a failure of primarily white adipose tissue and skeletal muscle to respond to insulin due to chronic high levels of glucose. Due to this insulin resistance, these tissues no longer effectively clear the blood of excess glucose. As developing countries continue to be influenced by a westernized lifestyle of high fat, high carbohydrate meals, and sedentary habits, it is predicted that the number of type II diabetics will continue to rise to epidemic proportions. Understanding the pathways involved in glucose homeostasis will provide more targets for pharmacological approaches to treating type II diabetes. In this context, the insulin signaling pathway and the molecular mechanisms underlying glucose uptake by adipocytes is a major field of interest.

Insulin Signaling

Increases in plasma blood glucose levels signal the β cells of the pancreas to release insulin which binds to receptors located on the surface of adipocytes and skeletal

muscle cells. Insulin binds to a tyrosine kinase receptor on the surface invoking autophosphorylation of its cytosolic tyrosine kinase domains. With these tyrosine kinase domains activated, the insulin receptor can subsequently phosphorylate various docking proteins in the signal transduction pathway. The insulin receptor substrate (IRS) family is the most common of these docking proteins. IRS phosphorylates other proteins such as phosphatidylinositol 3-kinase (PI3K) leading to the activation of Akt (also known as PKB) which has been linked to numerous downstream events such as vesicle translocation, gene transcription, and cytoskeletal remodeling (2-6, 10). Not all the potential downstream targets of Akt, however, have yet been identified.

One event triggered by insulin signaling in the adipocyte is the translocation of the internal stores of the facilitative glucose transporter, GLUT4, to the plasma membrane. Under basal conditions, GLUT4 is primarily localized in vesicles around the nucleus and these vesicles respond to insulin signaling by translocating and fusing with the plasma membrane resulting in increased glucose uptake (7) (Fig. 1)

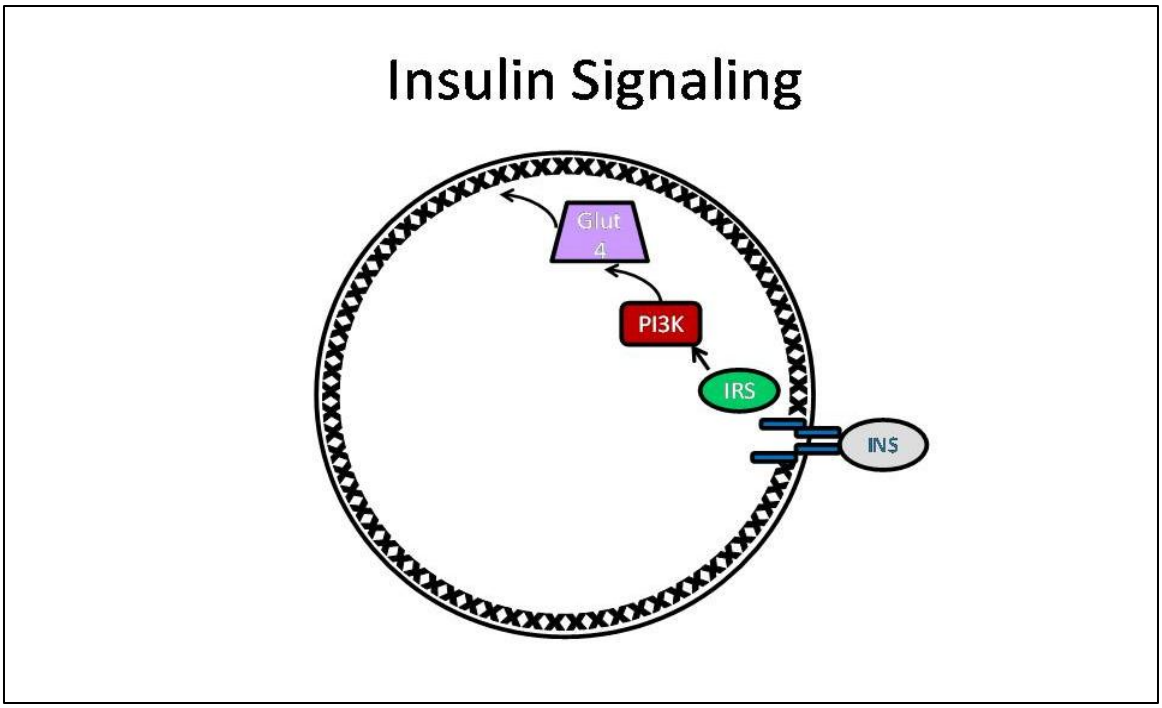

Figure 1. Representation of the insulin signaling pathway for glucose uptake in adipocytes.

The Role of the Cytoskeleton in Vesicle Trafficking

The cytoskeleton plays a necessary role in vesicle trafficking as well as in the docking and fusion of GLUT4 vesicles at the plasma membrane. In mature, differentiated adipocytes, filamentous (F) actin lines the inner surface of the plasma membrane. This cortical actin has been suggested to play a regulatory role in the processes of vesicle fusion and exocytosis by acting as a barrier to docking and fusion sites at the plasma membrane (3, 4, and 10). The insulin signaling pathway has been shown to induce the reorganization of this cytoskeletal barrier which is vital to GLUT4 vesicle fusion and thus GLUT4-mediated glucose uptake (2). The regulatory

mechanisms guiding this reorganization that have become a recent target of research. Motor proteins are thought to be a link in this regulation by allowing the actin filaments to contract. Recent studies have postulated that non-muscle myosin II is required for the cortical actin to reorganize and allow GLUT4 access to the plasma membrane to facilitate glucose uptake in the adipocyte (8, 14). Adipocytes have previously been shown to express two isoforms of non-muscle myosin II: myosin IIA and myosin IIB (8).

Myosin II

Non-muscle myosin II is a hexameric protein made up of two heavy chains, two regulatory light chains (RLC), and two essential light chains (ELC) (Fig. 2). The two heavy chains are structurally comprised of a globular head, a α-helical coiled coil middle region, and a non-helical tail region. The globular head region is the site of ATP binding and also the actin binding regions. Phosphorylation of serine 19 on the RLC induces the activity of the ATPase and hence contraction of the actin filaments (13).

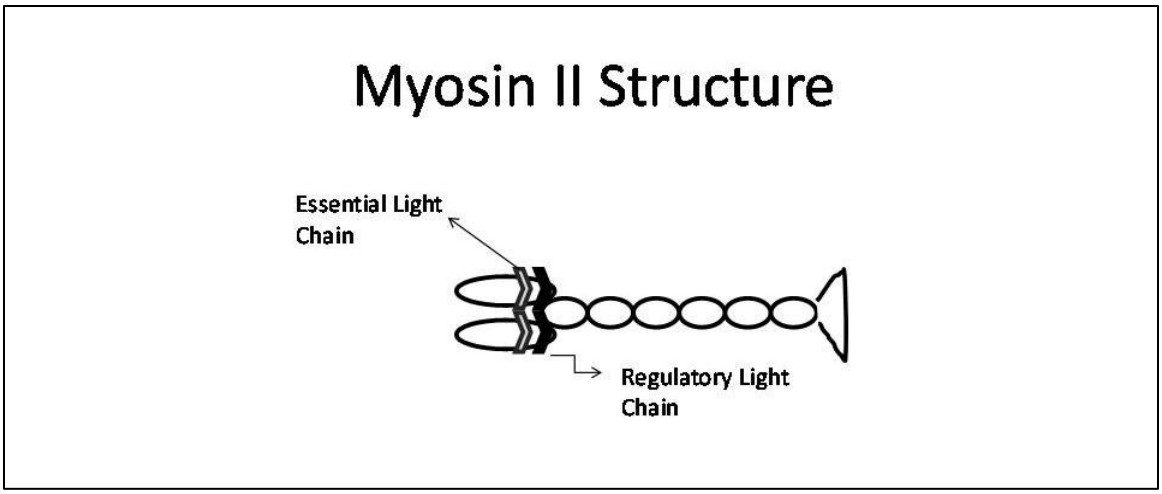

Figure 2. Representation of Myosin II Structure. Myosin II is composed of 2 heavy chains with globular heads (white), 2 regulatory light chains (black), and 2 essential light chains (gray) (13).

The two isoforms of myosin II in adipocytes interact with heavy chains encoded by different genes. The localization of the myosin II isoforms have been found to be species-and cell type-specific suggesting that the isoforms play distinct regulatory roles depending on cell type (9,12,25-27). In bovine arterial endothelial cells the myosin IIA and IIB isoforms have distinct spatial arrangements within the cell, which are altered when the cell undergoes migration, suggesting the localization patterns are indicative of specialized functions in the these cells (12). siRNA has been used to show that the localizations as well as the functions of the myosin II isoforms may differ depending on cell type (11, 26, 27).

Possible Role for Myosin II in Glucose Uptake in Adipocytes

In 3T3-L1 adipocytes, myosin IIA and IIB have different localization patterns under basal and insulin-stimulated conditions suggestive of diverse roles (8). Confocal microscopy has revealed that myosin IIB is localized at the cell cortex under basal conditions and does not appear to change its localization upon insulin stimulation. Myosin IIA, however, has a perinuclear localization under basal conditions and then translocates to the cortex upon insulin stimulation (8). Interestingly, this is similar to the localization pattern of GLUT4 vesicles (4) and suggests a possible role for myosin IIA in GLUT4 vesicle trafficking (8). Treatment of adipocytes with blebbistatin (a chemical inhibitor that binds to the motor domain of non-muscle myosin II resulting in inhibition of ATPase activity) resulted in significant impairment of insulin stimulated glucose uptake without affecting Akt phosphorylation or the translocation of GLUT4 vesicles (8). This suggests that myosin IIA may play an important role in glucose uptake by affecting the end process of GLUT4 vesicle fusion with the plasma membrane (8).

Although myosin IIA and GLUT4 translocate independently to the plasma membrane, myosin IIA activity is necessary for the fusion of GLUT4 vesicles to the membrane and also for the intrinsic activity of GLUT4 to allow the process of glucose uptake (14). Using confocal microscopy, both GLUT4 and myosin IIA were shown to co-localize at the plasma membrane only after insulin stimulation, consistent with an interactive role in the process of glucose uptake in the adipocyte. In addition, myosin IIA co-immunoprecipitates with GLUT4 only after insulin stimulation in adipocytes (14).

In our lab we have recently used chemical inhibitors to prevent the activation of myosin II, revealing its potential role in the glucose uptake process in adipocytes (14). Chemical inhibitors such as blebbistatin inhibit myosin II activity but this inhibition is not selective for the individual isoforms. Since adipocytes express both myosin IIA and IIB, it is important to distinguish the roles that the two isoforms play in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. My research used the technique of small interfering RNA (siRNA) for post-transcriptional gene silencing (PTGS) to specifically and effectively knockdown the expression of the myosin II isoforms. I hypothesized that myosin IIA is necessary for GLUT4-mediated glucose uptake upon insulin stimulation in adipocytes, while myosin IIB is not a major factor in insulin stimulated glucose uptake.

Possible Role for MLCK in Insulin-stimulated Glucose Uptake

With evidence of a critical role for myosin IIA in glucose uptake, the mechanisms regulating the activation of this protein are critical. Myosin II is regulated by phosphorylation of the RLC, and MLCK is known to phosphorylate the RLC.

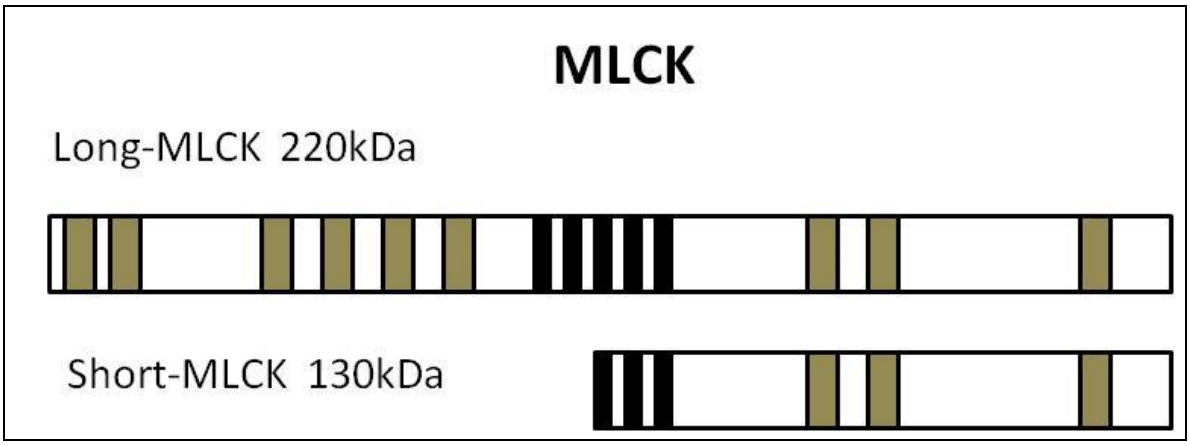

Figure 3. Representation of sequence motifs of the long and short isoforms of smMLCK. The long 220kDa isoform has 2 additional D(V/F)XXR repeats and 6 Ig motifs (30).

MLCK is a calcium/calmodulin-dependent (CaM) kinase most notably known for its role in activating myosin II to effect smooth muscle contraction. There are three known human genes that code for MLCK. Mylk2 and mylk3 code selectively for skeletal and cardiac tissue respectively. Mylk1, also known as the smooth muscle MLCK, is expressed in all adult tissues (36). Mylk1 encodes 3 different protein products from one open reading frame. The 220 kDa isoform is also known as the non-muscle MLCK. It contains the full sequence of the other two proteins plus an additional amino-terminal tail region that is associated with extended protein-protein interactions to actin filaments. The other two proteins are the 108/130 kDa and the 17 kDa kinase related protein (KRP) also known as telokin (31).

While several kinases are known to phosphorylate the RLC of Myosin II, only inhibition of MLCK has been shown to result in impaired phosphorylation of the RLC upon insulin stimulation. Interestingly, RLC is also the only known substrate for MLCK

(8, 11-14). Rho kinase has also been found to phosphorylate the RLC of myosin II. Previous studies have suggested that Rho kinase activity is limited to the interior of the cell and MLCK acts along stress fibers in the cell and along the periphery (30) suggesting a role for MLCK in an exocytic process. Previous studies in our lab revealed that chemical inhibition of MLCK but not Rho kinase significantly impaired insulin-stimulated glucose uptake (14).

Our lab has also shown that it is specifically MLCK that phosphorylates myosin IIA as a result of insulin signaling. Our lab was able to show that the MLCK inhibitor ML-7 [1-(5-iodonaphthalen-1-sulfonyl) homopiperazine] prevented the phosphorylation of the RLC associated with myosin IIA, impairing its translocation to the membrane. The chemical inhibition of myosin IIA with blebbistatin (an inhibitor of myosin II ATPase activity) and more specifically the inhibition of MLCK with ML-7 dramatically decreased the level of GLUT4 vesicle fusion at the membrane in the presence of insulin and resulted in a significant decrease in glucose uptake in adipocytes (14).

In order to determine the role of MLCK in the activation of myosin IIA, I used siRNA to knockdown expression of MLCK. I hypothesized that as a result of insulin signaling, MLCK phosphorylates the RLC of myosin IIA and results in GLUT4-mediated glucose uptake in adipocytes.

Possible Role for ERK2 in Insulin-stimulated Glucose Uptake

While insulin is known to activate the PI3K pathway, it also stimulates many other intracellular pathways such as the Mitogen-Activated Protein Kinase (MAPK) pathway. One of the leading targets of this transduction pathway is the extracellular-signal regulated kinases 1 and 2(ERK1/ERK2) though activation of the RAS, RAF, MEK cascade. MEK is an immediate upstream activator of ERK1/2. Previous studies have shown that in the presence of a constitutively active MEK (MAPKK) there is an increase in phosphorylation of ERK1/2, MLCK, and RLC (42). ERK1/2 has been shown to directly phosphorylate MLCK initiating its activation (42). It was also determined that ERK2 in the absence of MLCK expression was not sufficient to phosphorylate the RLC of myosin II (42). The MEK inhibitor, U-0126, not only decreases phosphorylation of ERK1/2 but also impairs insulin-stimulated glucose uptake in adipocytes giving further evidence of the MAPK pathway in glucose uptake (28).

Another substrate for ERK1/2 is the insulin receptor substrate-1 (IRS-1) which is upstream of PI3K in the insulin signaling pathway. Phosphorylation on serine 612 of IRS-1 by ERK inhibits further insulin signaling by providing negative feedback for this pathway mitigating chronic activation by insulin (39). Since their discovery, the two ERKs were thought to have redundant functions due to their sequence similarity and co-expression in a myriad of tissues. ERK1 and ERK2 knockout mice, however, have distinctly different phenotypes. ERK2 knockout mice die as embryos while ERK1 knockout mice have only slight developmental defects (40). In each knockout line, expression of the remaining ERK does not increase, suggesting these proteins are not

interchangeable (40). A study using short hairpin RNA in mouse embryonic fibroblasts to knockdown expression of ERK1 found that these cells appeared to increase proliferation while those with a decrease in ERK2 expression had a decrease in proliferation (46). In addition, a study using mouse C_2C_{12} muscle cells found that it was specifically ERK2 levels that increased upon leptin stimulation and not ERK1 (50). As well, the inhibition of ERK2 using the MEK inhibitor, PD98059, resulted in a reduction of insulin-stimulated glucose uptake as well as a reduction in GLUT4 recruitment to the plasma membrane (50). These results suggest that it is ERK2 that plays a major role in glucose uptake.

I hypothesized that it is specifically ERK2 that plays a necessary role in insulin-stimulated glucose uptake by the activation of myosin IIA via the phosphorylation of MLCK. I predicted that cells deficient in ERK2 protein would have impaired insulin-stimulated glucose uptake.

Possible Link Between CaMKIIdelta , MLCK, and ERK1/2 Regulation

CaM kinase II has also been suggested to have a regulatory role in the activation of MLCK. In vitro studies have shown MLCK to be phosphorylated by CaMKII (37).

CamKII is an oligomeric protein comprised from 4 different genes that code for individual subunits-α, β, γ, and δ. The α and β subunits are primarily expressed in neuronal tissue and the γ and δ subunits are expressed in a variety of tissues (35). The holoenzyme can be made up of monomers of the same or differing isoforms allowing for a large range of possible compositions (49). CaMKII is a serine/theonine kinase that

belongs to the same family of Ca2+/CaM dependent kinases as MLCK. CaMKII has been found to be activated by the binding of calcium/calmodulin initiating its autophosphorylation. Due to CaMKII's stacked structure, there is constitutive activation though this autophosphorylation of the kinase long after transient levels of calcium have decreased. It is this calcium-independent state that has led some researchers to suggest a more permissive role in insulin-stimulated glucose uptake than a regulatory role (35).

Studies using chemical inhibitors of CaMKII such as KN62 as well as other studies using calmodulin inhibitors have implicated a possible role for CaMKII in insulin-stimulated glucose uptake. A recent study using a purine analog inhibitor to CaMKII as well as cells expressing a mutant form of CaMKII revealed a significant decrease in insulin-stimulated glucose uptake (35). This inhibitor did not have a negative effect on the phosphorylation status of IR, IRS-1, p38MAPK, or Akt indicating a necessary role for CaMKII downstream of Akt activation (35). While the insulin signaling events preceding Akt phosphorylation are well defined, the substrates for Akt are still unknown.

CaMKII has also been linked to the regulation of ERK1/2 by way of its activation of RAF-1 upstream of MEK and ERK activation. It has recently been shown that CaMKII inhibition resulted in decreased glucose uptake (39). However, this study was performed for a 30 minute time point only, and it has been suggested that a longer time course study might have found that inhibition of CaMKII would result in an increased level of glucose uptake. It has been proposed that CaMKII plays a central role in

regulating the signaling events of ERK1/2. It is possible that in response to a constitutive signal CaMKII will actually inhibit glucose uptake by way of activation of ERK1/2 negative feedback loop (39).

With this in mind the siRNA technique was again used to down-regulate expression of CaMKIIδ to determine its role in regulating myosin IIA activation and insulin-stimulated glucose uptake. I hypothesized that inhibition of CaMKIIδ expression would inhibit myosin IIA phosphorylation by way of decreased MLCK phosphorylation and thus insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

CHAPTER II

MATERIALS AND METHODS

Materials

The following materials were obtained for this study. Cell culture media Dulbecco's Modified Eagle Medium High Glucose 1X Cat # 11995 was purchased from Gibco. Cell culture 15 cm plates were purchased from Greiner Bio-One Cat # 662160. Electroporation Gene Pulser cuvettes with a 0.4 cm electrode gap were obtained from BioRad Cat # 165-2088. Insulin was obtained from bovine pancreas and purchased through Sigma-Aldrich St.Louis,MO Cat # 15500-1G 016K1248, as well as dexamethasone , 3-isobutyl 1-methyl-xanthine (MIX) Sigma Cat# I5879, and Anti-Rabbit Secondary antibody from Sigma Cat # A 6154. Myosin IIA and IIB antibody was purchased from Covance Cat # PRB-440-P and from Cat # PRB-445-P respectively. The following antibodies were obtained through Cell Signaling: Actin Cat # 4968, ERK1/2 Cat # 9102, and P-ERK Cat # 9101S. MLCK and GLUT4 antibody was obtained from Santa Cruz Cat # 22223 and #1608 respectively. P-MLCK antibody was purchased from Invitrogen Cat # 441085G. CaMKIIdelta antibody was received as a generous gift from Dr. Singer's lab from Albany Medical College, New York.

Cell Culture

3T3-L1 fibroblasts were grown to confluency and then induced to differentiate using 10% Fetal Bovine Serum (FBS) / Dulbecco's Modified Eagle Media (DMEM) with the addition of 0.52 mM 3-isobutyl 1-methyl-xanthine (MIX), 1.7 µM insulin, and 1 µM

dexamethasone (DEX) on Day 0 (23). On Day 2, the media was changed to 10%FBS/DMEM plus 0.425 µM Insulin. Every two days thereafter the media was replaced with DMEM containing 10% FBS (23, 24).

siRNA Duplexes

The siRNA was purchased from Dharmacon, Inc. using ON-TARGET plus Smart POOL (see Appendix A for target sequences).

Electroporation

Day 8 3T3-L1 adipocytes were washed with a 1X Phosphate Buffered Saline (PBS) and solubilized using 0.5% trypsin-EDTA. After a 6 min incubation at 37°C and 5% CO_2, cells were resuspended in 10% FBS/DMEM and centrifuged for 5 min at 100 X g. The supernatant was removed and the cells were resuspended in 1 X PBS and centrifuged for 5 min at 100 X g twice. Finally, the cell pellet was resuspended in 1 ml 1 X PBS per 15 cm plate used. Cuvettes (4 mm gap) containing 1 nmol of the siRNA (water, Myosin IIA, Myosin IIB, Scramble, MLCK, CaMKIIδ , or ERK2) were prepared and placed on ice. A volume of 500 µl containing 2.5 x 10^6 cells along with the 1 nmol of siRNA was electroporated at 0.18 kV and 975 microfarads using a Bio-Rad Gene Pulser II. Immediately after electroporation, cells were re-suspended in 8.5 ml of 10%FBS/DMEM and the upper layer of lipid debris was removed using a pipette tip. After a 10 minute incubation period at room temperature, cells were re-plated onto multi-well plates. After 24 h, media was replaced with 10% FBS/DMEM (21, 22).

Glucose Uptake Assay

Electroporated 3T3-L1 adipocytes were re-plated onto 6-well plates and given a 48 h recovery period. The cells were then serum starved for a period of 4 h followed by 2 washes with 37°C Krebs Ringer Phosphate Buffer (KRP) pH7.4 containing 1.65 mM $CaCl_2$, 1.25 mM $MgSO_4$, 4.69 mM KCl, 5 mM Na_2HPO_4, and 130 mM NaCl. The cells were incubated in 950 µL of KRP/well for 10 min in a shaking water bath at 37°C. The designated treatment wells underwent insulin stimulation for 20 min with the application of 10 µl of insulin per well for a total concentration of 100 nM followed by the addition of 50 µl of a Glucose Solution containing KRP, 0.5 M glucose, and ^{14}C-deoxy-glucose. Total exposure of the cells to ^{14}C-deoxy-glucose was 10 min. Wells were washed twice with 1 X PBS at 4°C and lysed with a 0.5 M NaOH/0.1% SDS solution and then counted. The quantity of ^{14}C-2-deoxy-glucose uptake was assayed as disintegrations per min per mg protein and normalized to 1 representing basal levels (23).

Immunoblot Analysis

Electroporated 3T3-L1 adipocytes were re-plated onto 6 cm plates and given a 48-72 h recovery period. The cells then underwent a 4 h serum starvation period followed by 2 washes with KRP at 37°C. The wells designated for insulin treatment received 1 µl/1 ml KRP per well for a final insulin concentration of 100 nM and incubated at 37°C and 5% CO_2 for 20 min Cells were washed with 1 X PBS and then solubilized in a lysis buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM NaPPi, 2% Glycerol, and 1% NP-40) containing10 X Phosphatase cocktail (1:10) and protease inhibitors (1:100). The proteins were gently rocked for 20 min then centrifuged for 20

min at 4°C. The supernatant was collected and protein concentration was determined using the BSA Protein Concentration Standard Curve Spectrophotometer software. Samples were mixed with a 5 X Laemmli buffer and boiled for 5-10 min at 95°C then separated by 10% SDS-PAGE. The proteins were transferred to an Immobilon filter and then incubated for an h in a blocking solution of 1 X TTBS and 4% BSA. The primary antibody (1:1000) was diluted in this blocking solution and the filter was incubated at 4°C overnight while gently rocking. After 3 washes with 1 X TTBS, the filters were incubated in a solution containing the secondary antibody (1:2000) and 1 X TTBS for 45 min at room temperature. Filters underwent 3 washes with 1 X TTBS and were developed using the enhanced chemiluminescent (ECL) detection kit.

Immunofluorescence

Coverslips were mounted onto slides using a 50% glycerol solution. The slides were viewed using the Olympus IX81 Motorized Inverted Confocal Microscope and Fluoview FV500 software.

Optical Densitometry

Protein band intensity was quantified by densitometry using Quantity One software. Immunoblots were probed for myosin IIA and IIB and normalized to actin levels.

CHAPTER III

RESULTS

Determination of Transfection Efficiency

In order to optimize our electroporation protocol, we first wanted to determine transfection efficiency. 3T3-L1 adipocytes were electroporated with a siGLO transfection indicator targeting cyclophilin-B. Cyclophilin-B, also known as peptidylprolyl isomerase B, is commonly expressed in most tissues and cell viability is unaffected by its resultant knockdown with siRNA. These fluorescent oligonucleotides allow for visual assessment of transfection efficiency with the aid of confocal microscopy. Mature 3T3-L1 adipocytes were electroporated with either a vehicle (water) or increasing concentrations of the siGLO (Figure 4). Our results show there was a significant increase in transfection efficiency of the siGLO at the 500 nM concentration compared to the lower concentrations indicating a minimum of 500 nM for optimum transfection. These results suggest that our technique of transfecting cells with siRNA was successful.

Next we wanted to determine if we could impair expression of myosin IIA using siRNA. Mature 3T3-L1 adipocytes were electroporated with siRNA for Myosin IIA using increasing concentrations. Proteins were then collected at day 2 and 3 after electroporation of the siRNA to determine both the optimal concentration and the optimal time point for knockdown. In order to ensure even loading of protein samples on the SDS-PAGE, immunoblots were probed with an antibody against actin. As a control,

scramble oligonucleotide sequences were also electroporated at various concentrations to verify specificity of siRNA knockdown. The scramble sequences have no murine gene target thereby providing a negative control for the study. Consistent with a known half-life of 72 h for myosin IIA, it was determined that optimal experimental conditions for decreased protein expression peaked at 72 h (Figure 5). Determining the concentration and time point required for optimal knockdown was critical so that we could investigate the effect of specific genes on glucose uptake in 3T3-L1 adipocytes.

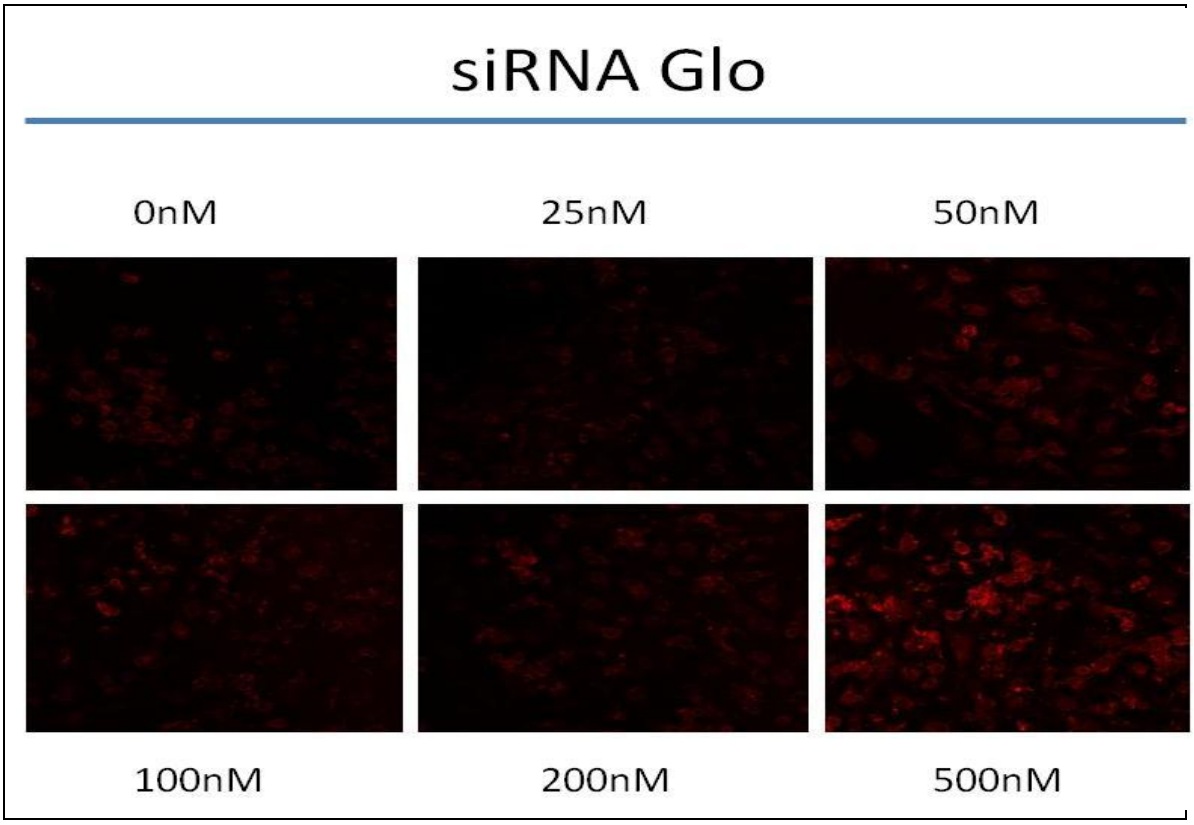

Figure 4. Determination of transfection efficiency using siRNA GLO. Mature 3T3-L1 adipocytes were electroporated with increasing concentrations of the transfection indicator siRNA Glo. Coverslips were fixed 24hours after transfection and assessed for transfection efficiency using confocal microscopy.

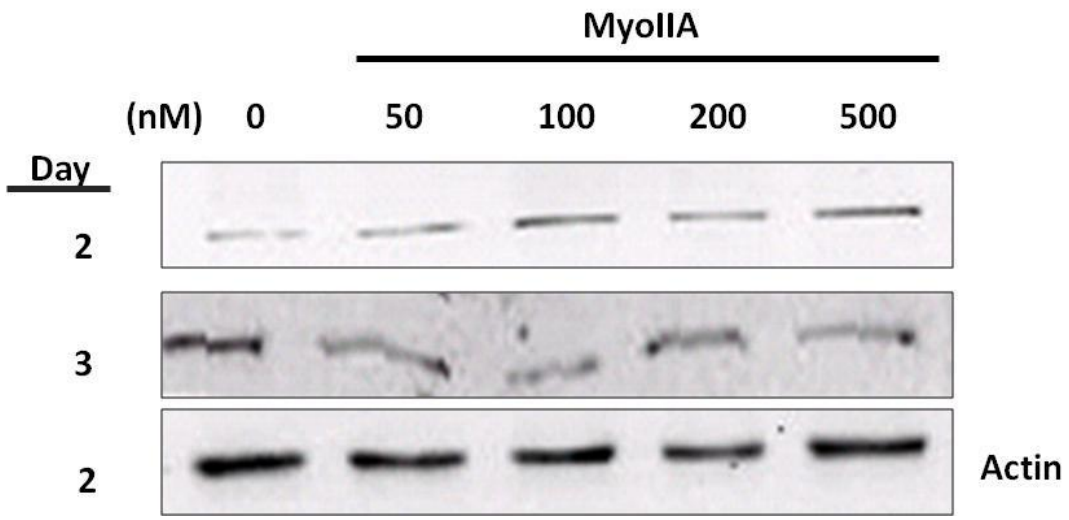

Figure 5. Reduced myosin IIA protein expression with increasing concentrations of siRNA. Following electroporation with various myosin IIA siRNA concentrations, proteins were collected on days 2 and 3 and assayed for myosin IIA or actin protein levels determined by Western blot analysis. Protein levels were normalized by comparison with actin levels.

Knockdown of Protein Levels of the Myosin II Isoforms with siRNA Transfection

Myosin IIA and myosin IIB have heavy chains that are encoded by isoform specific genes. 72 h after transfection of fully differentiated adipocytes with the target specific siRNA, protein levels of myosin IIA, myosin IIB, and actin were examined. Scramble siRNA were used as a control. Western blots were performed to determine knockdown of the target protein in relation to actin. It was determined by optical densitometry that protein levels of myosin IIA were reduced by ~60% and myosin IIB by ~50% to that of either the vehicle or the scramble electroporated cells. The cells electroporated with siRNA for myosin IIA did not have a decrease in myosin IIB expression. However, it was also observed that the myosin IIB siRNA knocked down the expression of the myosin IIB as well as myosin IIA equally, suggesting a shared

sequence region was targeted by the siRNA instead of a region specific to myosin IIB (Fig. 6).

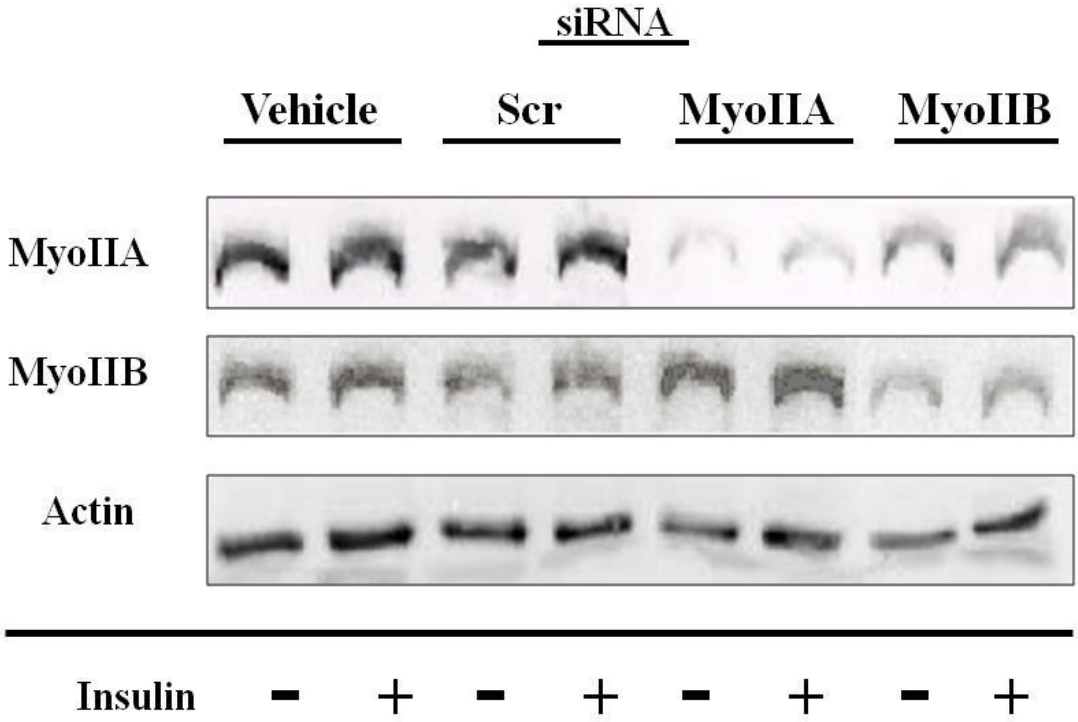

Figure 6. Decreased levels of myosin IIA and IIB following siRNA transfection. 3T3-L1 adipocytes were either electroporated with vehicle (water) or with 1 nmole of siRNA (Scramble, MyoIIA, or MyoIIB). After 72 h cells were serum starved for 4 h and then either left untreated or stimulated with insulin (100 nM) for 20 min Whole cell lysates were prepared, subjected to SDS-PAGE and then immunoblotted using antibodies against myosin IIA, myosin IIB or actin.

Knockdown of Myosin IIA Impairs Insulin-stimulated Glucose Uptake in 3T3-L1 Adipocytes

Previously, we have shown that the myosin IIA isoform is involved in insulin-stimulated glucose uptake. We next wanted to determine whether the knockdown of the myosin IIA isoform would impair insulin-stimulated glucose uptake in adipocytes. Glucose uptake assays were performed with [1-^{14}C]-2-deoxy-D-glucose to assess the effect of myosin IIA and myosin IIB knockdown on glucose uptake in adipocytes. There was no noticeable effect on glucose uptake under basal conditions with the knockdown of either myosin IIA or IIB compared to the vehicle or scramble (Figure 7). Cells transfected with a vehicle (water), scrambled non-coding sequence, or siRNA specific to either myosin IIA or myosin IIB were stimulated with 100 nM insulin for 20 min along with [1-^{14}C]-2-deoxy-D-glucose and assessed for [1-^{14}C]-2-deoxy-D-glucose uptake. Glucose uptake was decreased by approximately 30% for myosin IIA-deficient cells as compared to the vehicle. There was also a noticeable fold decrease in glucose uptake in cells deficient in myosin IIB, however as observed from the western blot analysis, both myosin IIB and myosin IIA protein levels were knocked down in the cells making these results less conclusive (Figure 7).

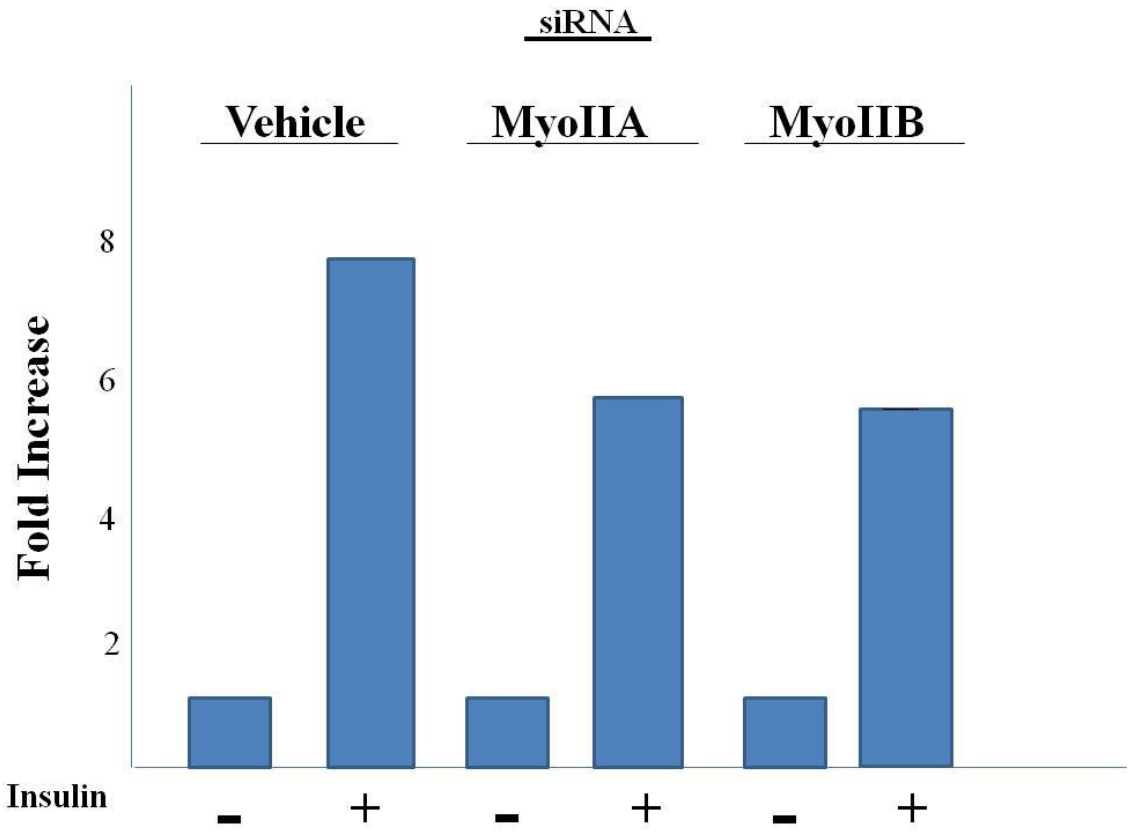

Figure 7. Knockdown of Myosin IIA impaired insulin-stimulated glucose uptake. Mature 3T3-L1 adipocytes were electroporated with vehicle (water) or with 1 nmole of the target siRNA (Scramble, MyoIIA, or MyoIIB). After 72 h cells were serum starved for 4 h and then either untreated (Basal) or stimulated with insulin (100 nM) for 20 min, followed by the addition of [1-^{14}C]-2-deoxy-D-glucose (0.1µCi/well) and 5 mM glucose for an additional 10 min. Cells were then lysed and glucose uptake calculated as disintegrations per mg protein.

Insufficient Knockdown of MLCK

We have previously shown that pharmacological inhibition of MLCK but not Rho kinase had an inhibitory effect on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. (14) To further support this finding, mature adipocytes were electroporated with siRNA specific for MLCK. Western blot analysis was performed to verify the

knockdown of MLCK. In non-muscle cells MLCK has the molecular weight of 220kDa and in smooth muscle cells MLCK is 150kDa. While I was not able to locate a band at either the 220 or 150 kDa sizes, a smaller band was detected. According to product description for the use of the MLCK antibody and personal communication with Dr. Anne Bresnick of Albert Einstein College of Medicine, there is a proteolytic fragment of 64 kDa. Using an MLCK antibody as well as a phospho-MLCK antibody, we detected the 64 kDa protein breakdown product. Our results show that MLCK knockdown was unsuccessful (Figure 8). However, we observed that the phosphorylation status of MLCK was noticeably increased upon insulin-stimulation compared to basal conditions giving further evidence of a role for MLCK in insulin-stimulated glucose uptake (Figure 8).

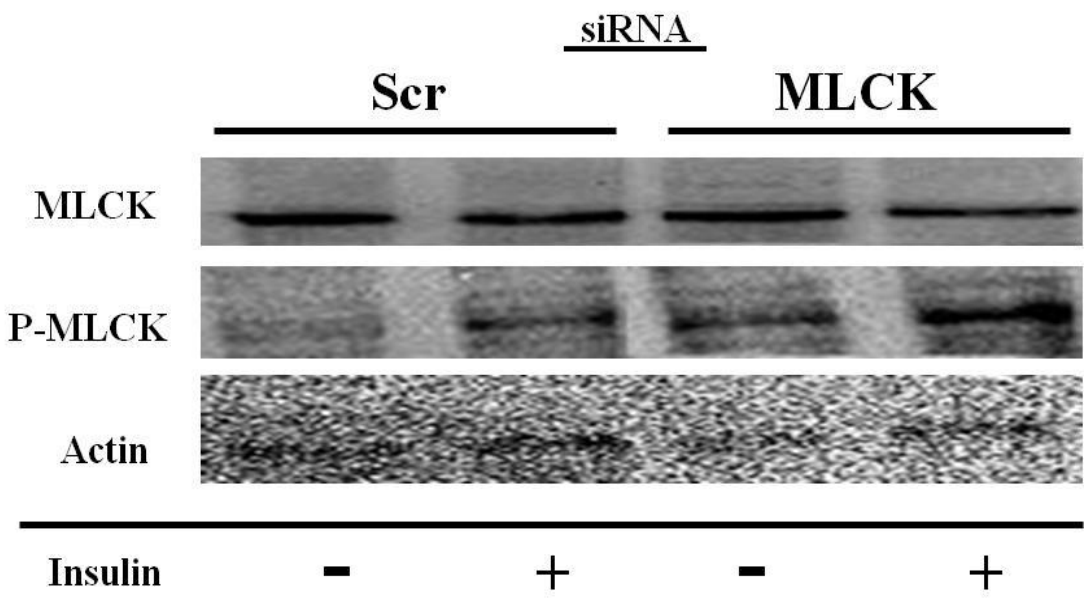

Figure 8. No change in protein expression levels of MLCK following siRNA transfection. 3T3-L1 adipocytes were electroporated with siRNA (1 nmole) targeting either MLCK or a scrambled sequence (Scr). After 48 h cells were serum starved for 4 h and then either left untreated or stimulated with insulin (100 nM) for 20 min. Whole cell lysates were prepared, subjected to SDS-PAGE and then immunoblotted using antibodies against MLCK, P-MLCK, or actin.

As expected, with no apparent decrease in protein expression of MLCK, there was no change in insulin-stimulated glucose uptake in cells electroporated with siRNA specific to MLCK compared to cells electroporated with a scramble sequence of siRNA (Figure 12). It is possible that a higher concentration of siRNA targeting the mRNA sequence for this gene is needed for sufficient knockdown.

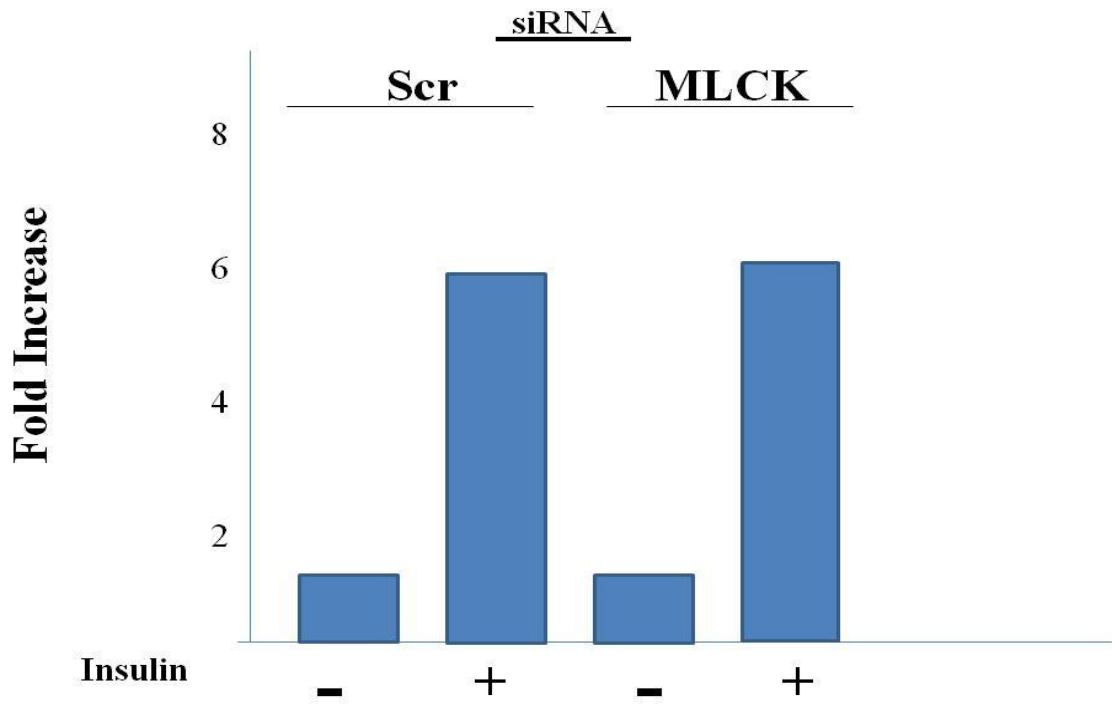

Figure 9. No effect in glucose uptake in insulin-stimulated cells subjected to MLCK siRNA transfection. Adipocytes were electroporated with 1 nmole of either MLCK or a scrambled sequence (Scr). Adipocytes were serum starved for 4 h and then either untreated (Basal) or stimulated with insulin (100 nM) for 20 min. Cells were then incubated in a solution containing [1-^{14}C]-2-deoxy-D-glucose (0.1µCi/well) and 5 mM glucose for an additional 10 min. Cells were lysed and glucose uptake calculated as disintegrations per mg protein.

siRNA Induced Inhibition of ERK2 Expression

ERK1 and 2 have been shown to have increased phosphorylation upon insulin signaling. ERK activation has also been found to induce the phosphorylation status of RLC, however ERK alone was not sufficient for this role (42). In a previous study ERK2 specifically was found to induce phosphorylation of MLCK and that in the absence of MLCK ERK2 was unable to phosphorylate RLC (42). I propose that ERK mediates the

phosphorylation of RLC by way of MLCK activation. It has also been suggested that ERK1 and 2 have distinct cellular functions and are not redundant. To assess the role of ERK2 specifically in this signal transduction pathway, I transfected adipocytes with siRNA targeting ERK2 and then assayed glucose uptake 48h after electroporation. As determined by immunoblot analysis (Figure 10), ERK2 levels were markedly reduced following siRNA treatment in comparison to protein levels in cells electroporated with a scramble siRNA. In addition, our results show that insulin-stimulated phosphorylation of MLCK was markedly reduced in cells electroporated with ERK2 siRNA compared to that of cells electroporated with a scramble sequence. This suggests that ERK2 is an upstream activator of MLCK.

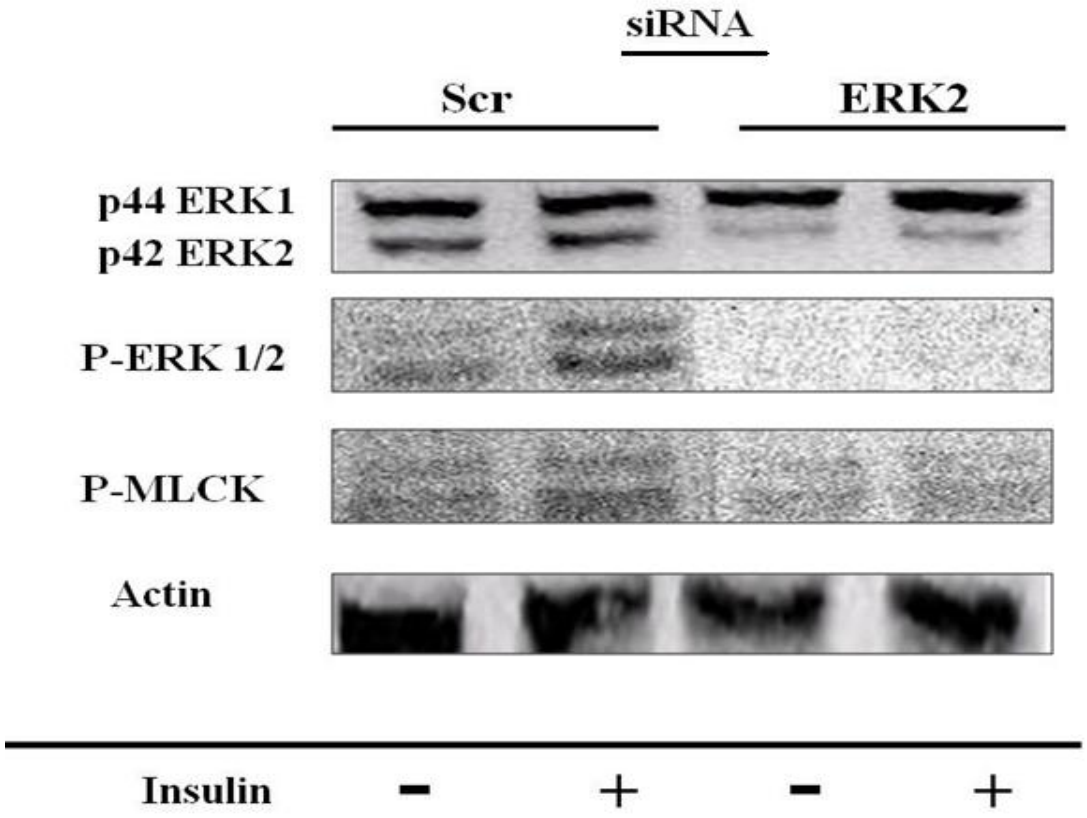

Figure 10. siRNA treatment effectively decreased protein levels of ERK2. 3T3-L1 adipocytes were electroporated with siRNA (1 nmole) targeting either ERK2 or a scrambled sequence (Scr). After 48 h adipocytes were serum starved for 4 h and then either left untreated or stimulated with insulin (100 nM) for 20 min. Whole cell lysates were prepared, subjected to SDS-PAGE and then immunoblotted using antibodies against either an ERK antibody (that detects both p44 ERK1 and p42 ERK2), P-ERK, P-MLCK, or actin.

Knockdown of ERK2 Impairs Insulin-stimulated Glucose Uptake in Adipocytes

Next, we wanted to determine the impact the reduced level of ERK2 would have on insulin-stimulated glucose uptake in the adipocytes. Figure 12 reveals no noticeable difference in the basal levels of glucose uptake into the cells with reduced levels of ERK2 compared to the control. However, under insulin-stimulated conditions, ERK2 deficient cells have a 20% decrease in glucose uptake compared to scramble. These results are suggestive of a role for ERK2 in insulin-stimulated glucose uptake in the adipocyte.

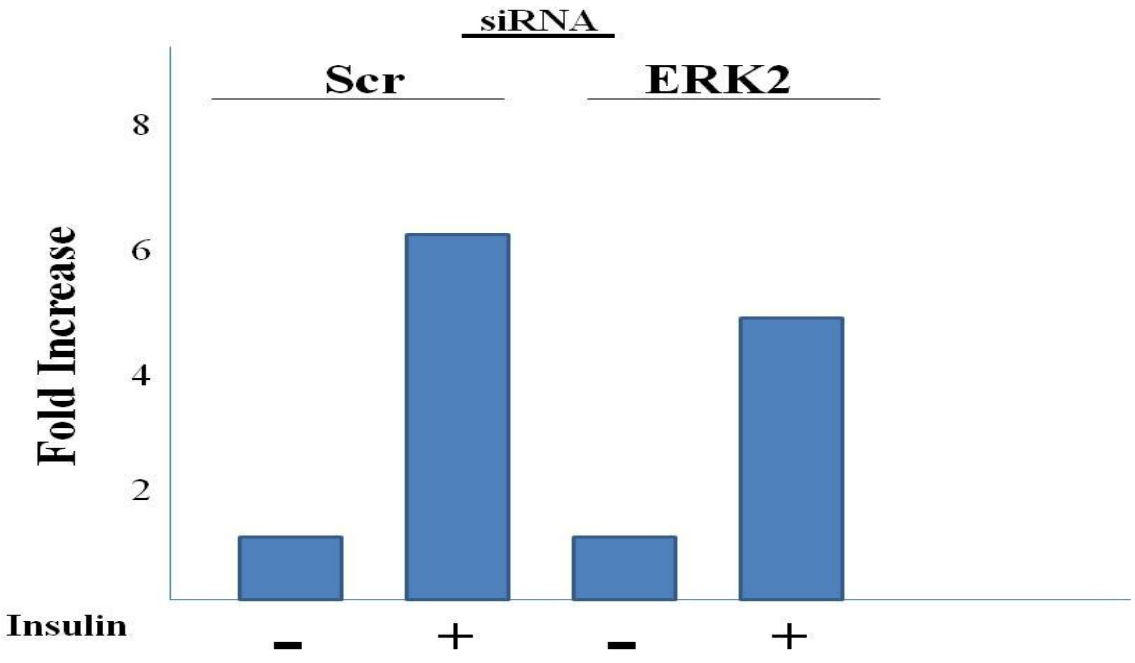

Figure 11. ERK 2 deficient cells have impaired glucose uptake in insulin-stimulated cells. Adipocytes were electroporated with 1 nmole of either ERK2 or a scrambled sequence (Scr). Adipocytes were serum starved for 4 h and then either untreated (Basal) or stimulated with insulin (100 nM) for 20 min. Cells were then incubated in a solution containing [1-^{14}C]-2-deoxy-D-glucose (0.1µCi/well) and 5 mM glucose for an additional 10 min. Cells were lysed and glucose uptake calculated as disintegrations per mg protein.

Knockdown of CaMKIIδ mRNA Expression

We also examined the role of CaMKIIδ in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Inhibitor studies, using the CaMKII inhibitor, KN62, have implicated a role for CaMKII in insulin-stimulated glucose transport. CaMKII has multiples substrates, and it has been suggested to play a role in the regulation of MLCK and ERK. With this in mind, we next wanted to explore a role for CaMKIIδ as a potential upstream regulator during insulin-stimulated glucose uptake. 3T3-L1 adipocytes were electroporated with oligonucleotides targeting CaMKIIδ which is the isoform prevalent in non-muscle cells. After 72 h, cell lysates were collected and subjected to SDS-PAGE and immunoblot analysis. Our results show that there was a reduced level of CaMKIIδ expression in adipocytes electroporated with CaMKIIδ siRNA compared to those with the scramble sequence (Figure 13). It was also observed that levels of phosphorylated MLCK were not inhibited upon insulin stimulation in cells with CaMKIIδ siRNA and were similar to those of cells treated with a scramble siRNA (Figure 13).

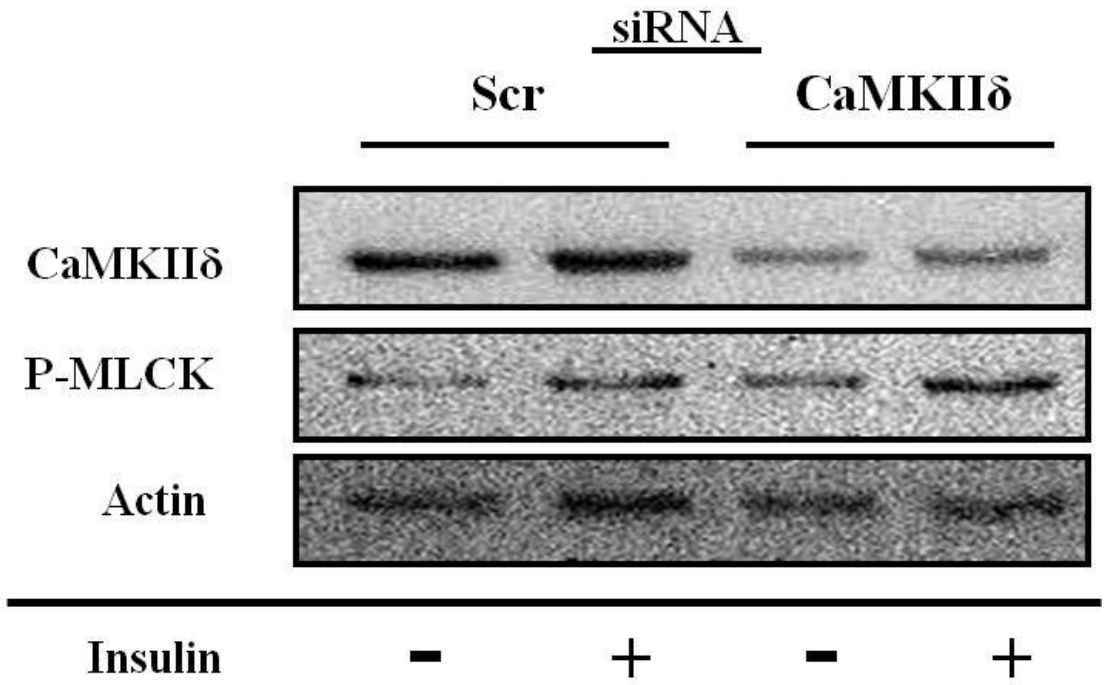

Figure 12. siRNA treatment effectively decreased protein levels of CaMKIIδ. 3T3-L1 adipocytes were electroporated with siRNA (1 nmole) targeting either CaMKIIδ or a scrambled sequence (Scr). After 72 h adipocytes were serum starved for 4 h and then either left untreated or stimulated with insulin (100 nM) for 20 min. Whole cell lysates were prepared, subjected to SDS-PAGE and then immunoblotted using antibodies against either CaMKIIδ, P-MLCK, or actin.

CaMKIIδ Knockdown Did Not Alter Insulin-stimulated Glucose Uptake

To investigate if CaMKIIδ plays a role in insulin-stimulated glucose uptake, a glucose uptake assay was performed on cells electroporated with a scramble sequence as well as cells electroporated with CaMKIIδ siRNA. As seen in Figure 14 the levels of glucose uptake remained constant under basal conditions for both treatments. Under insulin-stimulated conditions there appeared to be relatively no change in glucose uptake

which suggests that the CaMKIIδ isoform might not be a factor in insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

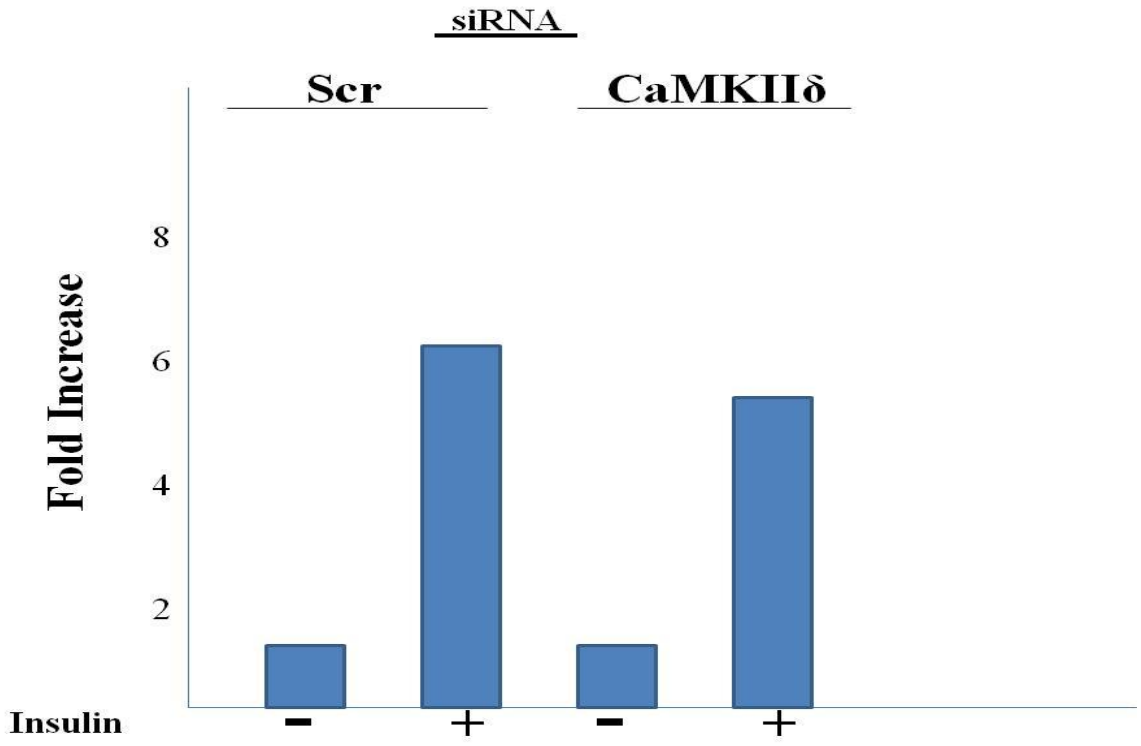

Figure 13. CaMKIIδ deficient cells have no impact on glucose uptake in insulin-stimulated cells. Adipocytes were electroporated with 1 nmole of either CaMKIIδ or a scrambled sequence (Scr). Adipocytes were serum starved for 4 h and then either untreated (Basal) or stimulated with insulin (100 nM) for 20 min. Cells were then incubated in a solution containing [1-^{14}C]-2-deoxy-D-glucose (0.1µCi/well) and 5 mM glucose for an additional 10 min. Cells were lysed and glucose uptake calculated as disintegrations per mg protein.

CHAPTER IV

DISCUSSION

Recently, myosin II has been implicated in GLUT4-mediated glucose uptake in adipocytes. To better understand the mechanism(s) involved in this process, the different myosin II isoforms A and B were knocked down using siRNA to allow distinct roles of the isoforms to be explored. While previous studies have used chemical inhibition, these inhibitors do not allow for the distinction between the two isoforms and the possibility remains that these methods might result in non-specific effects. My results are the first to reveal that inhibition of myosin IIA though the use of isoform-specific siRNA resulted in impaired insulin-stimulated glucose uptake. With the subsequent decrease of expression of myosin IIA in the cells treated with siRNA targeting myosin IIB, it provides evidence that myosin IIB does not have a role in this process since there was no further decrease in insulin-stimulated glucose uptake when both myosin IIA and IIB were knocked down.

I next wanted to examine the activation of myosin IIA. While several kinases are known to activate the RLC of myosin II, inhibition studies have shown that it is MLCK that is necessary for insulin-stimulated glucose uptake (14). I hypothesized that without MLCK, myosin IIA would not be able to mediate GLUT4-mediated glucose uptake. Unfortunately, siRNA treatment did not measurably knockdown expression of MLCK, nor were detectable changes observed in insulin-stimulated glucose uptake. It is

probable that a significantly greater decrease in MLCK expression would be necessary to impair insulin-stimulated glucose uptake. A recent study using rat hepatocytes treated with siRNA specific for MLCK found that not only was there a 70% reduction in expression of MLCK, but that there was also a reduction in cell proliferation (48). It is possible that knockdown of MLCK requires an increased concentration of siRNA or that the concentration I used was insufficient to reduce mRNA levels in a significant number of adipocytes. Western blot analysis was performed to verify protein knockdown; however we were unable to find a commercial antibody that would detect either the 220-MLCK or the 130-MLCK in 3T3-L1 adipocytes. Interestingly, a study found that cultured primary epithelial cells down-regulate the expression of the long and short isoforms of MLCK such that the 130 MLCK is virtually undetectable and that with continued passages only the 220 isoform was detectable (33). For future studies, it would be beneficial to try increasing oligonucleotide concentration and obtaining a more sensitive antibody for protein detection. Due to the speculation for distinct roles of the 220- and 130-MLCK isoforms, it might also be of interest to determine if adipocytes express both isoforms of MLCK and to specifically target the isoforms to distinguish if there is redundancy in function. Initially, my study had not focused on distinct isoform functions of MLCK. Oligonucleotides were obtained that targeted a region that was present in both the 220- and the 130-MLCK. While it would be difficult at this time to specifically knockdown the 130-MLCK since this sequence is also present in the 220-isoforms, siRNA sequences can be targeted to the additional amino tail region found only in the 220-MLCK. It is this additional amino tail region that is suggested to be a

continuous actin binding region for this isoform (34). In a recent study, the long-MLCK colocalized with actin bundles and also did not associate with either non-muscle myosin heavy chain IIA (NMHC-IIA) or NMHC-IIB; however the short MLCK appeared to have a perinuclear as well as peripheral localization pattern and was found to associate with NMHC-IIA (33). The 130 isoform did not seem to associate with either NMHC-IIB or with F-actin filaments (33). It is possible that the 130-MLCK phosphorylates myosin IIA perinuclearly stimulating myosin IIA to translocate to the plasma membrane and the cortically located 220-MLCK then phosphorylates myosin IIA triggering actin reorganization at the membrane. Recent studies have noted there is a distinct localization pattern for the long and short MLCK isoforms suggesting this redundancy in expression might be due to differential roles these isoforms have in cellular processes. A recent study determined that the 220 kDa and 130 kDa MLCK isoforms had particular localization patterns in bovine pulmonary arterial epithelial cells, smooth muscle cells, and fibroblasts (33).

While it is well established that insulin signaling activates the PI3K pathway, it is also known that insulin signaling stimulates many other transduction pathways. The MAPK pathway has also been linked to insulin-stimulated glucose uptake but the substrates involved with this pathway still need to be defined (28). This signaling pathway activates the RAS, RAF, MEK cascade of kinases. A downstream target of MEK is the ERK family of proteins. Recent studies have shown that ERK1 and 2 are able to directly phosphorylate MLCK and this activity led to increased phosphorylation of the myosin light chain (MLC) (42). Inhibitors of MEK along with siRNA knockdown

of ERK1 and 2 have resulted in reduced cell migration in COS-7 cells suggesting a role for ERK in cytoskeletal reorganization (42). Recently, evidence has revealed that ERK1 and 2 are not interchangeable but actually have distinct roles. With evidence linking an inhibition of ERK2 to reduced levels of insulin-stimulated glucose uptake and GLUT4 translocation in muscle cells, I hypothesized that it was specifically ERK2 that played a role in the metabolic process of glucose uptake (50). I hypothesized that ERK2 was responsible for the phosphorylation of MLCK and that reduced levels of ERK2 in 3T3-L1 adipocytes would result in impaired glucose uptake by way of reduced MLCK and RLC phosphorylation. My results reveal that with reduced ERK2 protein expression insulin-stimulated glucose uptake was impaired by 20% in comparison to control (scramble) levels. Basal levels of glucose uptake remained fairly constant in the cells treated with scramble as well as those with siRNA specific to ERK2. This reduced level of ERK2 appears to have resulted in a decrease in MLCK phosphorylation. For future studies, it would be interesting to determine if knockdown of ERK1 would lead to an increased level of insulin-stimulated glucose uptake. It has also been observed that localization of these ERK proteins change in response to different cellular processes. While I did not perform confocal or immunoprecipitation analysis of ERK1 and 2 for this study that could be an approach pursued in the future to determine their cellular localization in relation relation to MLCK.

While a role for ERK2 is becoming evident for insulin-stimulated glucose uptake in 3T3-L1 adipocytes, we were interested in determining an upstream regulator of ERK2. Studies have indicated a role for CaMKII both in the regulation of MLCK and in the

regulation of ERK1 and 2. While [Ca2+] intracellular have been found to increase upon insulin stimulation in the adipocytes, its role in the insulin signal transduction pathway is still unclear. CaMKIIδ is initially activated by Ca2+ levels but through autophosphorylation of its adjacent subunits, the kinase is able to remain activated long after intracellular calcium levels have decreased. It is possible that this allows CaMKII to regulate different substrates involved in the insulin signaling pathway. Recent studies have implicated a role for CaMKII in ERK activation. Studies using endothelial cells expressing a constitutively active CaMKII resulted in a significant increase in ERK activity (38, 45). Phosphorylation of ERK in these cells was inhibited by the CaMKII inhibitor, KN-62, and the MEK inhibitor U-0126 (38, 45). It was also shown that ERK phosphorylation was inhibited in the absence of activated CaMKII (38, 45). Other studies using CaMKII inhibitors have shown a decrease in phosphorylation of ERK in smooth muscle cells and endothelial cells (39, 45). However, in a recent paper that showed chemical inhibition of CaMKII resulted in a decrease in phosphorylated ERK; they were not able to find a subsequent reduction in MLC phosphorylation (45).

In addition to a role in the MAPK pathway, it is also possible that CaMKIIδ can phosphorylate the RLC of myosin II directly or by way of MLCK. Research exploring the unconventional myosin IC (Myo IC) in adipocytes has found that CaMKIIδ is able to phosphorylate Myo IC in vitro and subsequently activate its ATPase activity (43). It is important to note that this paper achieved a 90% reduction in CaMKIIδ expression and used a siRNA concentration of 20 nmole per 10 cm plate as opposed to the 2 nmole per 15 cm plate that we used in this study. It is possible that this greater reduction of

CaMKIIδ protein levels was required to result in an effect on glucose uptake, and the slight reduction of protein expression in my study was not sufficient to allow for conclusive results. While siRNA is a great tool for ascertaining gene function, a balance between toxicity and non-specific targeting needs to be maintained when determining the appropriate concentration of siRNA to use in each experiment.

CaMKII is a kinase with multiple substrates which may include ERK2 and MLCK. This is also a kinase with four isoforms with most cells expressing at least one of these (38). In this study the delta isoform was targeted as playing a possible role in insulin-stimulated glucose uptake due to a high expression of this isoform in adipocytes. Recent research has concluded that in a majority of cells the α, γ, and δ isoforms appear to have a nuclear localization pattern, while in neural tissue the β isoform co-localizes with the cytoskeleton (37). It would be beneficial to confirm what isoforms are expressed in 3T3-L1 adipocytes. It is possible that while the delta isoform is highly expressed in adipocytes; other isoforms are also expressed and have a potential role in insulin-stimulated glucose uptake. It is also possible that these other CaMKII isoforms can compensate for the loss of the delta isoform.

My results revealed that knockdown of the CaMKIIδ isoform did not inhibit insulin-stimulated glucose uptake in 3T3-L1 adipocytes. While knockdown of ERK2 had a noticeable decrease in insulin-stimulated glucose uptake, knockdown of CaMKIIδ did not significantly impair ERK phosphorylation (data not shown) or MLCK phosphorylation suggestive that this isoform of CaMKII does not play a role in insulin-

stimulated glucose uptake. The results of our study were able to show that we could successfully knockdown myosin IIA and B expression in adipocytes and that a reduction in myosin IIA protein levels in 3T3-L1 adipocytes resulted in impaired insulin-stimulated glucose uptake. We were also able to show that MLCK is phosphorylated upon insulin stimulation giving further evidence that this kinase is involved in insulin-stimulated glucose uptake. We were also able to show that ERK2 knockdown was achieved and resulted in a decrease in insulin-stimulated phosphorylation of MLCK and also impaired insulin-stimulated glucose uptake. We were also able to show we had a slight reduction in protein expression of CaMKIIδ which did not have a significant effect on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. These are significant findings in delineating a pathway involved in insulin-stimulated glucose uptake in adipocytes leading the way to future medicinal targets for type II diabetes.

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APPENDIX A: TABLES

siRNA duplexes ON-TARGET plus Smart POOL

Scramble	Myosin II A	Myosin II B
5'-UGGUUUACAUGUCGACUAA-3'	5'-GGGCUUAUCUACACCUAUU-3'	5'-GUAUUAAGUUUGCGAAGGA-3'
5'-UGGUUUACAUGUUGUGUGA-3'	5'-UUGUAGAGCUGGUAGAGAA-3'	5'-GGACAGAGCAAGAUAUUUU-3'
5'-UGGUUUACAUGUUUUCUGA-3'	5'-AUAAGAACCUGCCCAUCUA-3'	5'-GGGCAUCUCUGCUCGCUAU-3'
5'-UGGUUUACAUGUUUUCCUA-3'	5'-GCAGACAAGUACCUCUAUG-3'	5'-GGACUUAUCUAUACUUACU-3'

MLCK	CaMKIIδ	MAPK1/ Erk2
5'-GGUCUGUCCUCGCCAAGAA-3'	5'-GAUCAAGGCCGGAGCUUAC-3'	5'-GGAUACAGAUCUUAAAUUG-3'
5'-UGACGGAGCGGGAGUGUAU-3'	5'-GCUAGAAUCUGCCGUCUCU-3'	5'-UGAGAGGGCUAAAGUAUAU-3'
5'-CAGCCGAGCAGAUGGAUUU-3'	5'-CGACGAGUAUCAGCUCUUU-3'	5'-ACAAGAGGAUUGAAGUUGA-3'
5'-CGACUAGGAUCUGGGAAAU-3'	5'-GAAGCGGGAUGCCAAAGAC-3'	5'-UAUACCAAGUCCAUUGAUA-3'