FULCHER, FRANKLIN KENT, M.S. Regulation of Myosin II Isoform Localization and Activation during Insulin Stimulated Glucose Uptake in 3T3-L1 Adipocytes. (2006)
Directed by Dr. Yashomati M. Patel. 55 pp.

Insulin stimulated glucose uptake is a critical component of glucose homeostasis. The studies presented here show that myosin IIA is actively recruited to the plasma membrane upon insulin stimulation. I also show that inhibiting the interaction of myosin II with actin or inhibiting the activation of myosin II by myosin light chain kinase resulted in myosin IIA remaining localized to the perinuclear region of 3T3-L1 adipocytes. Based on my findings I hypothesized that the localization and activation of myosin IIA is regulated by the activation of the insulin signaling pathway. The studies presented here reveal underlying interactions and mechanisms affecting insulin stimulated glucose uptake providing important information that can be used to optimize current treatments or develop new treatments for type II diabetes.

REGULATION OF MYOSIN II ISOFORM LOCALIZATION AND ACTIVATION DURING INSULIN STIMULATED GLUCOSE UPTAKE IN 3T3-L1 ADIPOCYTES.

by

Franklin Kent Fulcher

A Thesis Submitted to
The Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2006

Approved by

_____

Committee Chair

Dedicated to Suzanne, Olivia and Adaline

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.

Committee Chair _____

Committee Members _____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to thank the following for their help and support: My advisor Dr. Yashomati M. Patel and committee members Dr. Dennis Lajeunesse and Dr. John Tomkiel for all of their help and support throughout my research, my supervisor and mentor Dr. Karen Katula, and the Faculty and Staff of the University of North Carolina at Greensboro Biology Department.

TABLE OF CONTENTS

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Type II diabetes mellitus is a major public health issue and is a contributing factor to many other health problems such as blindness, heart disease, renal failure and non-traumatic amputations. Type II diabetes accounts for 90 to 95% of all diagnosed cases of diabetes and affects more than 6% of the adult population in the US. Some contributing factors involved in the development of type II diabetes are aging, obesity, family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, physical inactivity, race and ethnicity. While aging is a contributing factor to the development of type II diabetes, recent studies have shown that there is a rise of type II diabetes in children. Recent reports from the American Diabetes Association indicate that up to 45% of children diagnosed with diabetes have type II diabetes.

Type II diabetes is a disease that affects blood glucose levels due to a decreased response to insulin. Type II diabetes begins as insulin resistance, a disorder in which the cells do not respond to normal insulin levels. As insulin levels increase to facilitate glucose uptake, the pancreatic beta cells cease to function properly and gradually lose the ability to produce insulin.

The prevalence of this disease in our society identifies a need to understand all the factors involved in the development of type II diabetes. Identifying factors on the molecular level will contribute to our further understanding of this disease state and the development of potential treatments.

This study is an investigation of the cellular mechanisms involved in insulin dependent glucose uptake. My previous studies have shown that insulin stimulated glucose uptake in mature 3T3-L1 adipocytes was impaired by the inhibition of non muscle myosin II activity [1]. My current investigation of myosin II isoforms and their role during insulin stimulated glucose uptake identifies underlying factors regulating the dynamic cellular processes responsible for glucose homeostasis. In turn these results could offer insight for new treatments related to type II diabetes.

Insulin stimulated glucose uptake

The binding of insulin to its receptor initiates a cascade of events critical for glucose uptake. The insulin receptor is a transmembrane tyrosine kinase receptor consisting of a series of two exofacial α subunits, which bind insulin and two plasma membrane bound β tyrosine kinase subunits. Activation of the receptor results in its auto-phosphorylation and the recruitment and phosphorylation of insulin receptor substrates (IRS). This then activates

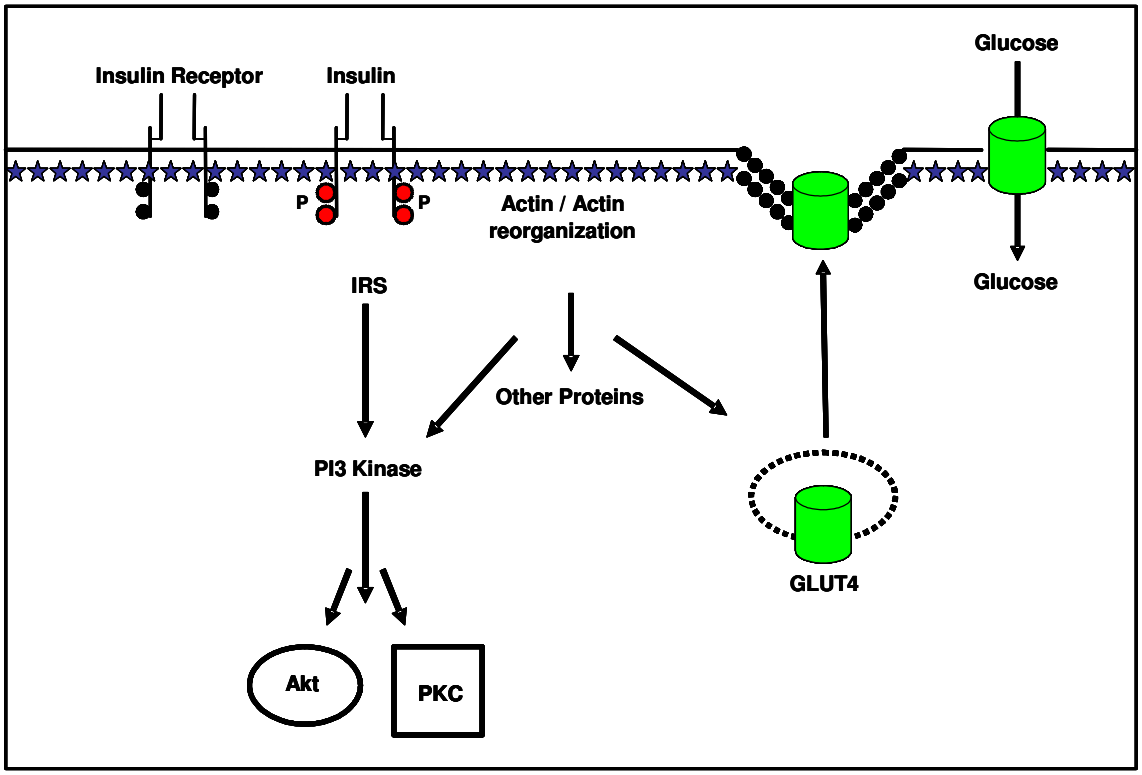

Figure 1. Representation of insulin stimulated GLUT4 translocation.

phosphatidylinositol-3 kinase (PI3 kinase) which activates both the serine / threonine protein kinase Akt / PKB along with the atypical protein kinase C (aPKC) [2, 4]. The activation of Akt / PKB and / or aPKC stimulates the translocation of vesicles containing the insulin responsive glucose transporter 4 (GLUT4) from intracellular "pools" to the plasma membrane (Fig. 1) [2, 4]. Insulin also activates other PI3 kinase independent pathways which facilitate glucose uptake for example the MAPK and Cbl pathways [2, 4, and 6].

A functional role for the actin cytoskeleton in adipocytes

Mature differentiated adipocytes do not contain a stress fiber matrix to provide structural support; instead mature adipocytes have a distinct layer of cortical actin adjacent to the plasma membrane [7]. The actin cytoskeleton in 3T3-L1 adipocytes consist of a network of filamentous actin (F - actin) [7]. The actin cytoskeleton gives the cell its structural integrity, but also acts as a network of tracks for vesicle transport and as a barrier to either endocytosis or exocytosis at the plasma membrane. The actin “tracks” have been shown to facilitate translocation of GLUT4 containing vesicles from intracellular pools to the plasma membrane in adipocytes [7]. One of the events that have to occur for GLUT4 vesicle docking and fusion is that the actin layer at the plasma membrane has to be reorganized. The structural integrity provided by the cortical actin layer is separated at specific regions at the plasma membrane to facilitate proper docking and fusion of GLUT4 vesicles to the plasma membrane [7]. In both muscle and adipose tissue the remodeling of cortical actin in has been shown to be regulated by the activation of the motor protein myosin II [8]. Investigation of myosin II and the actin cytoskeleton may further our understanding of GLUT4 vesicle fusion and thus, further our understanding of insulin stimulated glucose uptake.

Non-muscle myosin II

Non-muscle myosin II is a cytoplasmic myosin that is responsible for cytokinesis and the structural integrity of many cell types. Three isoforms of myosin II have been identified that carry out similar functions which do not overlap. Myosin II is expressed in most tissues in varying amounts although; in some tissues only one isoform is expressed such as chicken intestinal epithelium and human blood platelets [11, 25]. Previous studies have identified distinct patterns of myosin II isoform localization in several cell types, such as the localization patterns of myosin IIA and myosin IIB in the edge of migrating endothelial [11]. Some other relevant examples in other cell types are the localization of myosin IIA and IIB in cultured neurons at actin rich regions of neurite growth cones [26]. Another example is the localization of myosin IIA at dynamic regions of cytoskeletal compartments of osteoclasts where as the localization of myosin IIB remains somewhat constant throughout different osteoclast activation cycles [29].

Myosin II exists as a hexameric structure consisting of two heavy chains (MHC), two regulatory light chains (RLC) and two essential light chains (ELC) (Fig. 2). The heavy chains are structurally oriented to form a globular head domain that expresses binding sites for ATP and actin.

Myosin II motor activity and parallel filament assembly are regulated by the phosphorylation of residues localized on the RLCs [10, 24].

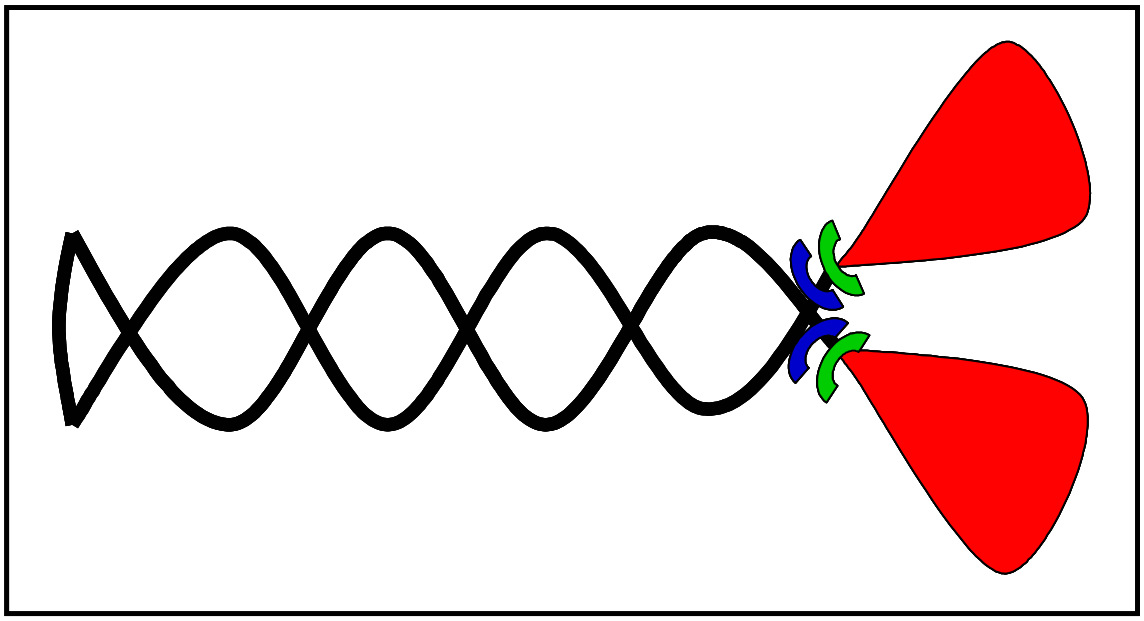

Figure 2. Representation of the myosin II hexameric structure. Two heavy chains in a coiled coil orientation, black. Two essential light chains, green, and two regulatory light chains, blue. Globular terminal head region, red, carboxy terminal tail. Adapted from [10] Current Opinions in Cell Biology

Three kinases have thus far been identified that phosphorylate the regulatory light chains of myosin II: the Ca^{2}+ / calmodulin dependent myosin light chain kinase (MLCK), Rho kinase (RHOK) and p21 activated kinase [10, 24]. Phosphorylation of the regulatory light chains in a Ca^{2}+ / calmodulin dependent manner via myosin light chain kinase is very specific in initiating the binding of myosin II to filamentous actin [33 - 36]. The regulatory light chain of myosin II is the only known substrate for myosin light chain kinase [10].

Myosin II and insulin stimulated glucose uptake

My recent studies have shown a direct link between myosin II function and insulin stimulated glucose uptake [1]. This was accomplished by utilizing the specific myosin II inhibitor, blebbistatin. Blebbistatin is a non competitive inhibitor of myosin II ATPase activity.

The data presented in Figure 3 is from a series of glucose uptake assays from my recent publication identifying a novel role for myosin II during insulin

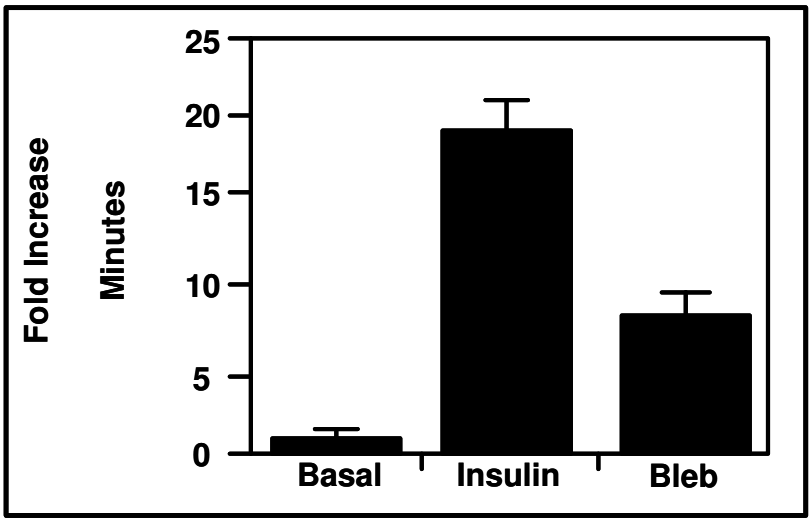

Fig. 3 Inhibition of myosin II impairs insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Adipocytes were serum starved in the presence of vehicle (0.1% DMSO) or 100 µM blebbistatin, and then stimulated with 100 nM insulin for 10 min and assayed for [14C]2-deoxy-d-glucose uptake in the presence of vehicle or 100 µM blebbistatin. Glucose uptake was calculated as disintegrations per mg protein and expressed as percent of the vehicle control. Results are means ± SEM of three independent experiments.

stimulated glucose uptake [1]. We observed approximately a 75 % inhibition of insulin stimulated glucose uptake in the blebbistatin group compared to the insulin controls. These findings are the first to identify a functional role for myosin II activity in insulin stimulated glucose uptake.

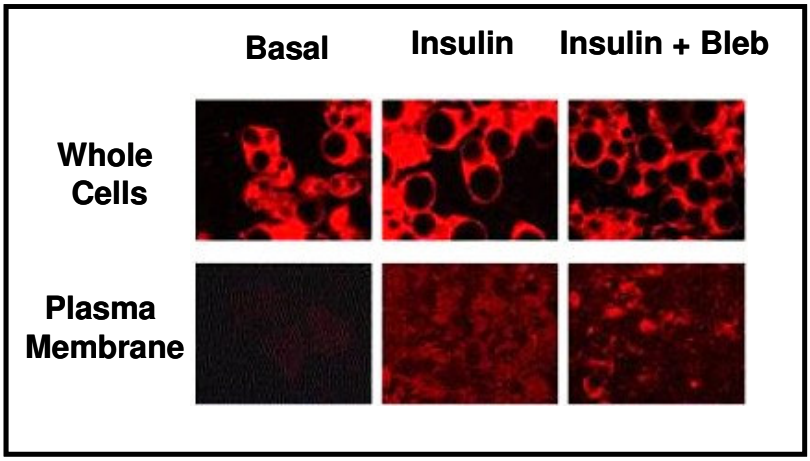

Figure 4. Insulin-stimulated GLUT4 translocation was not affected by treatment with blebbistatin. Adipocytes were treated according to the glucose uptake protocol as described in materials and methods, and GLUT4 protein was visualized by immunofluorescence using a GLUT4-specific antibody. GLUT4 localization was assayed in whole cells (top panel) as well as plasma membrane sheets (bottom panel) in the basal condition and after stimulation with insulin in the presence and absence of 100 μM blebbistatin. The results are representative images from three independent experiments.

Since GLUT4 translocation is critical for insulin stimulated glucose uptake, I wanted to determine whether GLUT4 translocation to the plasma membrane

during insulin stimulated glucose uptake was altered by inhibiting myosin II activity. 3T3-L1 adipocytes were incubated in the presence of 100 μm blebbistatin. The cells were then stimulated with insulin and assayed for GLUT4 localization using confocal microscopy (Fig. 4) [1]. My studies show that GLUT4 translocation to the plasma membrane was not affected by the inhibition of myosin II activity. GLUT4 vesicles can be qualitatively identified in the insulin control and insulin plus blebbistatin treatment (Fig. 4). When myosin II activity is inhibited, insulin stimulated glucose uptake is impaired yet GLUT4 vesicles still translocated to the plasma membrane. This suggests that myosin II is involved in some downstream event in GLUT4 mediated glucose uptake such as GLUT4 vesicle fusion with the plasma membrane. Previous studies have shown that contraction of the actin cytoskeleton in rat chromaffin cells and human MRC-5 fibroblast can lead to localized remodeling at the cellular cortex [30,31] The dynamic process of actin remodeling may be responsible for the docking and fusion of GLUT4 with the plasma membrane. Taken together these results reveal that there is a relationship between GLUT4 mediated glucose uptake and the activity of myosin II.

In my previous publication, two myosin II isoforms were identified in 3T3-L1 adipocytes by western blot analysis (Fig. 5). In other cell types where both isoforms have been shown to be expressed, distinct localization patterns have been identified where one isoform may only occupy a specific intracellular space

at a given time. An example of this pattern has been identified in migrating human MRC-5 fibroblasts where myosin IIA localized to the lamellipodia

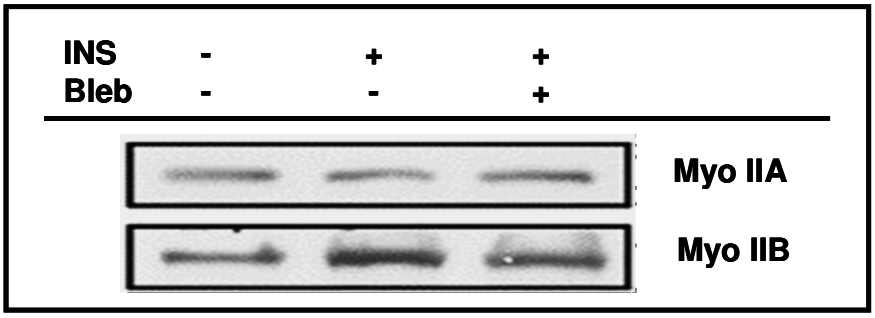

Figure 5. Effect of blebbistatin on myosin II levels in 3T3-L1 adipocytes. Cells were pre-treated with 0.1% DMSO vehicle or 100 µM blebbistatin and were then left untreated (basal) or stimulated with 100 nM insulin for 10 min. Cell lysates were analyzed by SDS-PAGE, blotted to membrane, and then probed with antibodies against myosin IIA, and myosin IIB, as indicated. The results are representative of three individual experiments

extensions and throughout the posterior region of the cells with myosin IIB. Similar patterns have been revealed in activated osteoclast where myosin IIA was shown to localize in cytoskeletal compartments of polarized and non-polarized osteoclast where myosin IIB was not found in these dynamic regions [29, 30]. Since two myosin isoforms have been identified in 3T3-L1 adipocytes it could be postulated based on previous findings in other cell types expressing both myosin IIA and IIB, that the two have distinct intracellular localization patterns during the dynamic process of insulin stimulated glucose uptake. With

the identification of two myosin II isoforms in 3T3-L1 adipocytes it is important to identify the localization patterns of the two myosin isoforms prior to and during insulin stimulated glucose uptake. It is also of interest to determine whether the myosin II isoforms are recruited to facilitate glucose uptake and which of the isoforms is being activated during insulin stimulated glucose uptake.

Based on previous findings revealing distinct patterns of myosin II isoform localization during dynamic cellular processes, it can be postulated that the same pattern of distribution may be revealed in 3T3-L1 adipocytes during insulin stimulated glucose uptake with insulin signaling regulating the process [10 – 12 and 30]. I hypothesize that myosin II localization and activity are regulated during insulin stimulated glucose uptake in 3T3-L1 adipocytes

Previous studies have shown isoform specific localization patterns of myosin IIA and B in various cell types [9 – 12 and 29 - 30]. In my previous work I identified that both myosin IIA and myosin IIB are expressed in 3T3-L1 adipocytes. Since both of these isoforms are expressed in 3T3-L1 adipocytes and inhibition of myosin II activity impairs insulin stimulated glucose uptake but not the recruitment of GLUT4 to the plasma membrane, it is important to determine where the isoforms are localized in the basal state and during insulin stimulation to understand their role during insulin stimulated glucose uptake [1, 24]. Based on the examples of the localization patterns of myosin II in other cell types expressing both isoform IIA and IIB I hypothesize that the IIA isoform will

be localized near the perinuclear region and the IIB isoform will be localized to the plasma membrane.

Insulin stimulation assays were conducted and myosin II isoforms were examined using isoform specific antibodies to either myosin IIA or myosin IIB. Confocal microscopy was utilized to determine the localization of the two isoforms in 3T3-L1 adipocytes prior to and after insulin stimulation.

I expect to see distinct localization patterns of the two isoforms in the basal state and insulin stimulated state based on previous studies in other cell types. I also predict that myosin IIA will be recruited to the plasma membrane upon insulin stimulation since previous studies have shown that myosin IIA can re-localize upon exposure to various stimuli [10]

My previous studies have shown that inhibition of myosin II activity significantly decreases GLUT4 mediated insulin stimulated glucose uptake although there is no effect on GLUT4 translocation to the plasma membrane (Fig. 3 and 4) [1]. In previous studies investigating the localization of myosin IIA and myosin IIB during dynamic cellular processes such as the migration of endothelial cells and activation stages of osteoclast, it has been shown that myosin IIA was localized primarily in dynamic regions of activity [25, 29]. If myosin IIA is recruited to the plasma membrane, a dynamic region of activity, it is of interest to determine whether myosin IIA is recruited prior to, with or after GLUT4 vesicle recruitment to the plasma membrane.

I predict that the recruitment of myosin II to the plasma membrane will be specific and not random. This prediction is based on my findings revealing that myosin IIA is actively recruited during insulin stimulated glucose uptake and findings from previous investigations revealing that myosin IIA is localized at specific regions in various cell types [9 - 11, 25, 26, 30 and 32]. I hypothesize that myosin IIA will be recruited to the plasma membrane at sites close to GLUT4 vesicles after the insulin signaling pathway is activated.

Using cultured 3T3-L1 adipocytes, confocal microscopy assays were performed to determine the localization of myosin IIA and GLUT4 at various time points after insulin stimulation. These assays reveal the temporal relationship between GLUT4 vesicle translocation and myosin IIA recruitment during insulin stimulated glucose uptake.

I expect to see specific and not random localization patterns of myosin IIA recruitment to the plasma membrane after insulin stimulation. I also predict that if myosin IIA is facilitating glucose uptake, then the recruitment of myosin IIA must be targeted to specific regions at the plasma membrane where GLUT4 has translocated to and not to random sites.

My previous work was the first to reveal that 3T3-L1 adipocytes express both myosin IIA and IIB isoforms [1]. Since both isoforms are expressed in 3T3-L1 adipocytes and inhibition of myosin II activity impairs glucose uptake it is important to identify if insulin is regulating the activity of one or both of the

isoforms. Myosin II activity is stimulated by the phosphorylation of the regulatory light chain. Phosphorylation of the RLC can be controlled by three kinases: myosin light chain kinase (MLCK), Rho kinase (Rhok) or the p21 activated kinase [10]. As shown in my previous study, insulin stimulated glucose uptake was dependent on myosin II activity [1]. I hypothesize that there will be a significant increase in the phosphorylation of the RLC of myosin II during insulin stimulation.

Activated myosin II was determined by immunoprcipitation assays. Myosin IIA and myosin IIB were immunoprecipitated and immunoblotted to detect the regulatory light chain and phosphorylation status of the regulatory light chain. Antibodies specific for myosin IIA, myosin IIB, RLC and phosphorylated RLC were used.

Based on findings from my previous study showing that the inhibition of myosin II impairs glucose uptake but not GLUT4 translocation, I predict that the regulation of myosin II activity via phosphorylation of the regulatory light chain is dependent on insulin signaling, following the proposed recruitment and localization patterns of myosin IIA I predict that myosin IIA activity will be regulated by insulin.

CHAPTER II

MATERIALS AND METHODS

Materials for this study were obtained from the following: Cell culture reagents were purchased from Gibco (Grand Island, NY). Insulin was obtained from Roche Diagnostics (Indianapolis, IN). Dexamethasone, 3-isobutyl-1-methyl-xanthine, Triton X-100, ML-7 and myosin IIA antibody was purchased from Sigma-Aldrich (St. Louis, MO). Blebbistatin was obtained from Calbiochem (San Diego, CA). GLUT4 antibody (C-20) and Ag + agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Myosin IIA and Myosin IIB antibodies were obtained from Covance (Berkeley, CA). Alexa Fluor 488 and 594, donkey anti-goat IgG, goat anti-rabbit IgG and Image-iT FX Signal Enhancer were purchased from Molecular Probes (Eugene, OR). Protein assay kit, Immuno - Star - AP chemiluminescent kit and Goat IgG_2 alkaline phosphatase conjugated antibody was obtained from Bio-Rad Laboratories (Hercules, CA). Mounting media was purchased from DAKO Cytomation (Carpinteria, CA

Cell culture

3T3-L1 pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS) until confluent and maintained for an additional 2 days (until day 0). Differentiation was induced on day 0 by the

addition of 0.5 mM methylisobutylxanthine (M), 0.5 µM dexamethasone (D), 10 µg / ml insulin (I), and 10% fetal bovine serum (FBS) in DMEM. On day 3, the MDI medium was replaced with DMEM/10% FBS, and changed every two days thereafter until analysis. Cytoplasmic triglyceride droplets became abundant between days 4 and 5, and by day 7, cells were fully differentiated [15].

Insulin stimulation assays and timed course insulin stimulation assays

3T3-L1 adipocytes were differentiated as previously described [15]. Adipocytes were cultured in 10 cm tissue culture plates for lysates analysis. 3T3-L1 pre-adipocytes were cultured in 10 cm plates containing cover slips to be used for confocal microscopy assays. Insulin stimulation assays were carried out on mature adipocytes using the following protocol: Mature adipocytes (day 9-12) were serum starved for 4 hours in the presence of 0.1% DMSO (vehicle) or 100 µM blebbistatin or 10 µM ML-7. Adipocytes were then washed twice with 37℃ Krebs–Ringer Phosphate (KRP) buffer (pH 7.4) containing 128 mM NaCl, 4.7 mM KCl, 1.65 mM $CaCl_2$, 2.5 mM $MgSO_4$, and 5 mM Na_2HPO_4, and then placed in KRP buffer containing vehicle or either 100 µM blebbistatin or 10 µM ML-7. Samples were untreated (basal) or treated with 100 µM insulin for 10 min. The time course insulin stimulation assays were treated as described above and stimulated with 100 µM insulin up to the following time points; 2 minutes, 4 minutes, 6 minutes and 10 minutes. Cells were washed 3 times with cold 1x PBS. Cells were either lysed or fixed as described below.

Immunofluorescent staining for confocal microscopy assays

Whole cell multiple immunostaining procedures were used to assay for the myosin II isoforms and GLUT4. Previously treated cells from the insulin stimulation assay were fixed in a 2% paraformaldehyde solution for 5 minutes and washed 3x with 1x PBS. The samples were then permeabilized with a 0.25% Triton X-100 solution for 5 min at 4 ^{C}. Following permeabliazation, the samples are washed 3x in 1x PBS. The samples were then incubated for 1 hour in a solution containing a GLUT4 antibody (Santa Cruz Biotechnology; Santa Cruz, CA) and myosin IIA antibody or myosin IIB antibody (Covance; Berkeley, CA). After the primary incubation the samples were washed 3x with 1x PBS. The cells were then sequentially incubated with an anti-goat and anti-rabbit Alexa-fluor 488 and 594 labeled antibodies (Molecular Probes; Eugene, OR). The samples were then washed 4x with 1x PBS. Samples were viewed using a FLUOVIEW FV5OO laser scanning confocal microscopy system. An image was collected at the top, center and base of the cells at specific regions of interest. The images were then compiled using FLUOVIEW software.

Immunoblot analysis

After insulin stimulation cells were washed three times in cold PBS and placed immediately in a lysis buffer containing 25 mM Hepes, pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 2% glycerol, 5 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaPPi, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg / ml

aprotinin, 5 mg / ml leupeptin, and 5 mg/ml pepstatin. Lysates were gently mixed for 20 min at 4℃ and then centrifuged at 6000*g* for 20 min at 4℃ to pellet insoluble material. The supernatants were retained for subsequent analysis. Protein concentrations of whole-cell lysates were determined using the Bradford dye binding method (Bio-Rad; Hercules, CA). Samples were then heated for 5 min at 95 C, separated by 12% SDS–PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting. Immunoblots were developed with an ECL kit and exposed to x-ray film for detection.

Immunoprecipitation

Antibodies were added at (3 µg / ml) to whole-cell lysates (1 mg) for each subsequent assay. Lysates were then agitated overnight (± 16 hrs) at 4℃. Agarose beads (protein A / G PLUS–agarose beads; Santa Cruz Biotechnology,) were mixed with the immunoprecipitates and agitated for 1 h at 4 ℃. Immunoprecipitates were recovered by centrifugation at 2500*g* and washed three times with ice cold lysis buffer. Immunoprecipitated proteins were then dissolved in 2× Laemmli buffer, heated for 5 min at 95 C, subjected to 10% SDS–PAGE, and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with the indicated primary antibodies and visualized by enhanced chemiluminescence.

CHAPTER III

RESULTS

Myosin II isoforms exhibit distinct localization patterns during insulin stimulated glucose uptake in adipocytes

Previous studies have revealed distinctive localization patterns for the two isoforms of myosin II (IIA and IIB) depending on cell type and cellular context [9 - 12, 25 and 26]. In my previous study I identified that 3T3-L1 adipocytes express both myosin IIA and IIB [1]. These studies determined first, the localization pattern of the myosin II isoforms in the basal state and also whether the observed patterns of isoform localization were altered in the presence of insulin. I conducted insulin stimulation assays and then examined the localization of myosin IIA and IIB using isoform specific antibodies and confocal microscopy

I observed that under basal conditions, myosin IIA is localized primarily to the perinuclear region in adipocytes but is recruited to the plasma membrane upon insulin stimulation (Fig. 6; A and B). In contrast, myosin IIB is localized to the cell cortex, and this pattern of localization was not altered upon insulin stimulation (Fig. 6; C and D). These results reveal first that there are distinct localization patterns of myosin II and that insulin signaling results in the recruitment of myosin IIA to the plasma membrane.

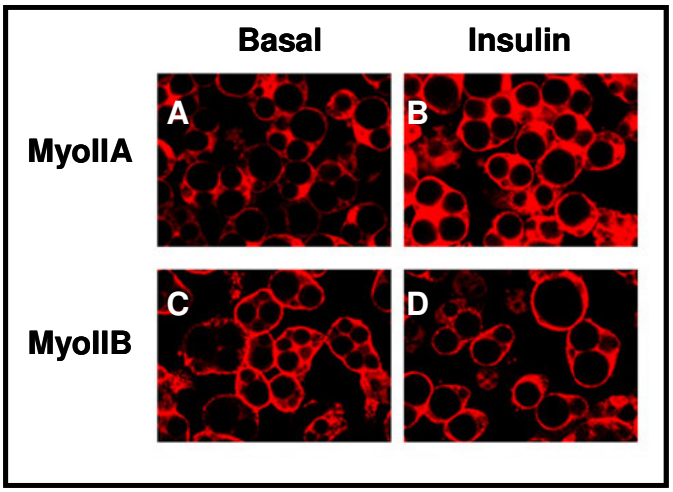

Figure 6. The effect of insulin stimulation on myosin II localization in adipocytes. 3T3-L1 adipocytes were treated according to the insulin stimulation protocol as described in materials and methods and the myosin II isoforms were visualized by immunofluorescence using either a myosin IIA or myosin IIB-specific antibody. Myosin IIA and myosin IIB localization was analyzed in the basal condition and after stimulation with insulin using confocal microscopy. The results are representative images from three independent experiments.

Myosin IIA is co-localized with GLUT4 vesicles at the plasma membrane after insulin stimulation in 3T3-L1 adipocytes

In previous studies myosin II has been shown to play an important role in vesicle trafficking by interacting with filamentous actin [16, 17]. Recruitment of myosin IIA from the perinuclear region to the plasma membrane suggests that there may be an interaction between myosin II and the cortical layer of actin

found in 3T3-L1 adipocytes. Myosin II at the plasma membrane could possibly be involved in the rearrangement of the actin fibers to allow the GLUT4 - containing vesicles to properly dock and fuse with the plasma membrane. Since both myosin IIA and GLUT4 vesicles translocate to the plasma membrane upon insulin stimulation, it was of interest to determine whether GLUT4 and myosin IIA translocate to the same or different areas of the plasma membrane.

Confocal microscopy was used to verify the recruitment of myosin IIA and GLUT4 from the perinuclear region to the plasma membrane. The localization patterns of myosin IIA and GLUT4 during insulin stimulation in 3T3-L1 adipocytes was also identified.

In the basal state myosin IIA localized to the perinuclear region with GLUT4 (Fig. 7; A-C), localization of myosin IIA and GLUT4 is clearly indicated in the merged image by the yellow coloration at the perinuclear region.
Insulin stimulation results in the translocation of myosin IIA and GLUT4 from the perinuclear region to specific regions of the plasma membrane (Fig 7; D and E).

The translocation of the two is indicated in the merged image (Fig. 7; F). The observed increase of yellow coloration at the plasma membrane indicates areas where myosin IIA and GLUT4 are localized at or near the same space.

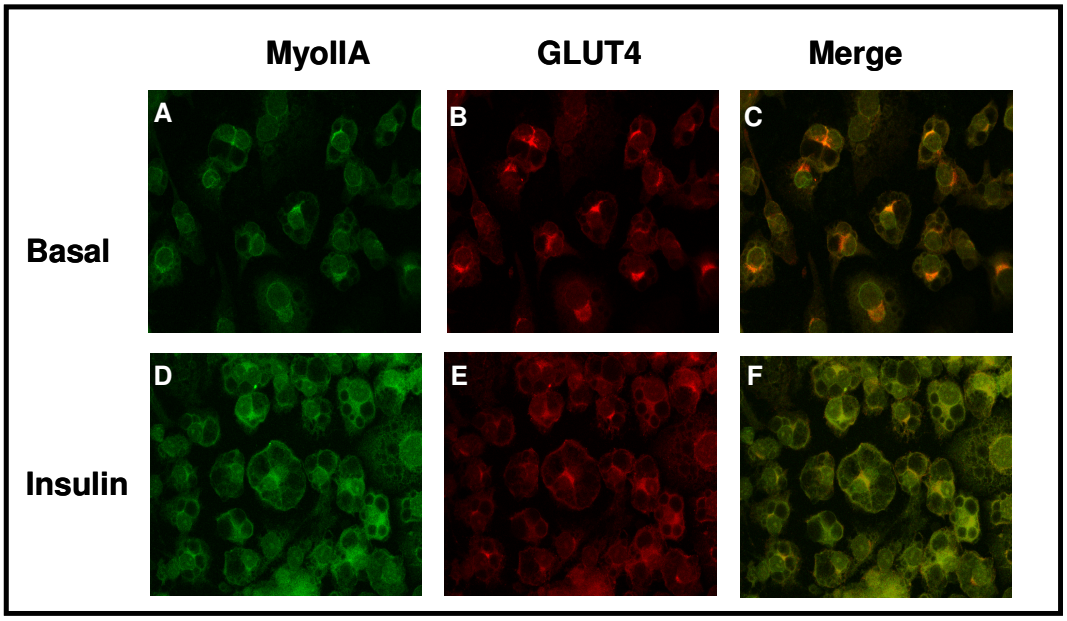

Figure 7. The effect of insulin stimulation on myosin IIA and GLUT4 localization in adipocytes. 3T3-L1 adipocytes were treated according to the insulin stimulation protocol as described in materials and methods. Myosin IIA and GLUT4 were visualized by immunofluorescence using antibodies specific to myosin IIA and GLUT4 respectively. Myosin IIA and GLUT4 localization was analyzed in the basal condition and after stimulation with insulin using confocal microscopy. The results are representative images from three independent experiments.

Myosin IIA is recruited after GLUT4 vesicles to the plasma membrane during insulin stimulated glucose uptake

Since both myosin IIA and GLUT4 are recruited from the perinuclear region upon insulin stimulation to specific points at the plasma membrane it is important to determine the timing of these events to answer which is recruited to the plasma membrane first.

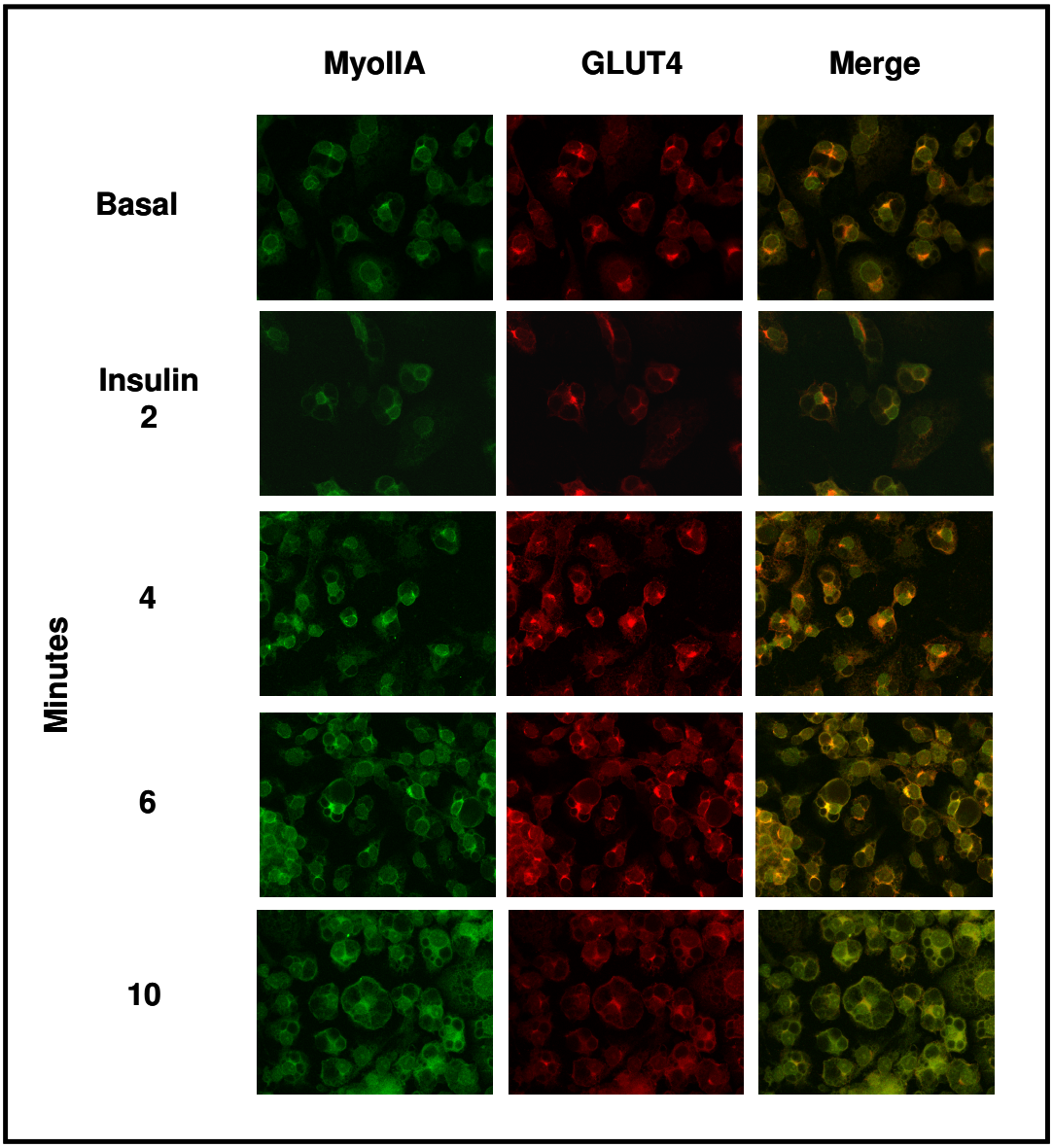

Figure 8. Recruitment patterns of myosin IIA and GLUT4 during insulin stimulation . 3T3-L1 adipocytes were treated according to the time course insulin stimulation protocol as described in materials and methods. Myosin IIA and GLUT4 were visualized by immunofluorescence using antibodies specific to myosin IIA and GLUT4. Myosin IIA and GLUT4 localization was analyzed in the basal condition and after stimulation with insulin at 2,4,6 and 10 minutes using confocal microscopy. The results are representative images from three independent experiments.

Confocal microscopy was used to identify patterns of co-localization of myosin IIA and GLUT4 during a time course insulin stimulation assay. The localization of myosin II and GLUT4 were determined at 0, 2, 4, 6 and 10 minutes after insulin stimulation.

Upon insulin stimulation, myosin IIA was recruited to the plasma membrane at approximately 4 minutes (Fig.8; 4 minutes). The amount of myosin IIA at the plasma membrane increased up to 10 minutes (Fig. 8; 6 and10 minutes). The amount of GLUT4 increased from 2 minutes up to 10 minutes (Fig. 8; 2-10 minutes). I have identified that myosin IIA is recruited after GLUT4 containing vesicles have translocated to the plasma membrane and GLUT4 recruitment was shown to be independent of the activity of myosin IIA. These findings suggest a role for myosin IIA in facilitating the fusion of GLUT4 vesicles at the plasma membrane.

Recruitment of myosin IIA to the plasma membrane is regulated by the insulin signaling pathway

Next, I examined how insulin was regulating the recruitment of myosin II to the plasma membrane by utilizing a series of inhibitors known to disrupt the activity of myosin II and the interaction of myosin II with actin. In my previous study I have shown that treating 3T3-L1 adipocytes with blebbistatin impaired insulin stimulated glucose uptake (Fig. 3) [1]. Blebbistatin inhibits the interaction of myosin II to actin by blocking the ATPase activity of myosin II. By blocking the

ATPase activity of myosin II it impairs the ability of myosin II to bind to filamentous actin.

Temporal assays were performed and confocal microscopy was used to identify the patterns of localization of myosin IIA and GLUT4 during a time course insulin stimulation assay. The localization of myosin II and GLUT4 were determined at 0, 2, 4, 6 and 10 minutes after insulin stimulation.

I observed that the recruitment of myosin IIA from the perinuclear region to the plasma membrane was impaired in cells that had been treated with blebbistatin (Fig. 9; 4-10 minutes). At the 10 minute time point the level of myosin IIA at the plasma membrane was significantly lower than that observed in the insulin control (Fig. 8; 10 minutes). As indicated by the minimal change in yellow coloration observed at the plasma membrane in the blebbistatin treatment merged images that blebbistatin does in fact disrupt the recruitment of myosin IIA during insulin stimulated glucose uptake.

To identify how insulin was regulating the recruitment of myosin IIA another known inhibitor of myosin II activity was used. ML-7 inhibits the phosphorylation of the RLC of myosin II by impairing the activity MLCK. [10, 24, 27 and 28].

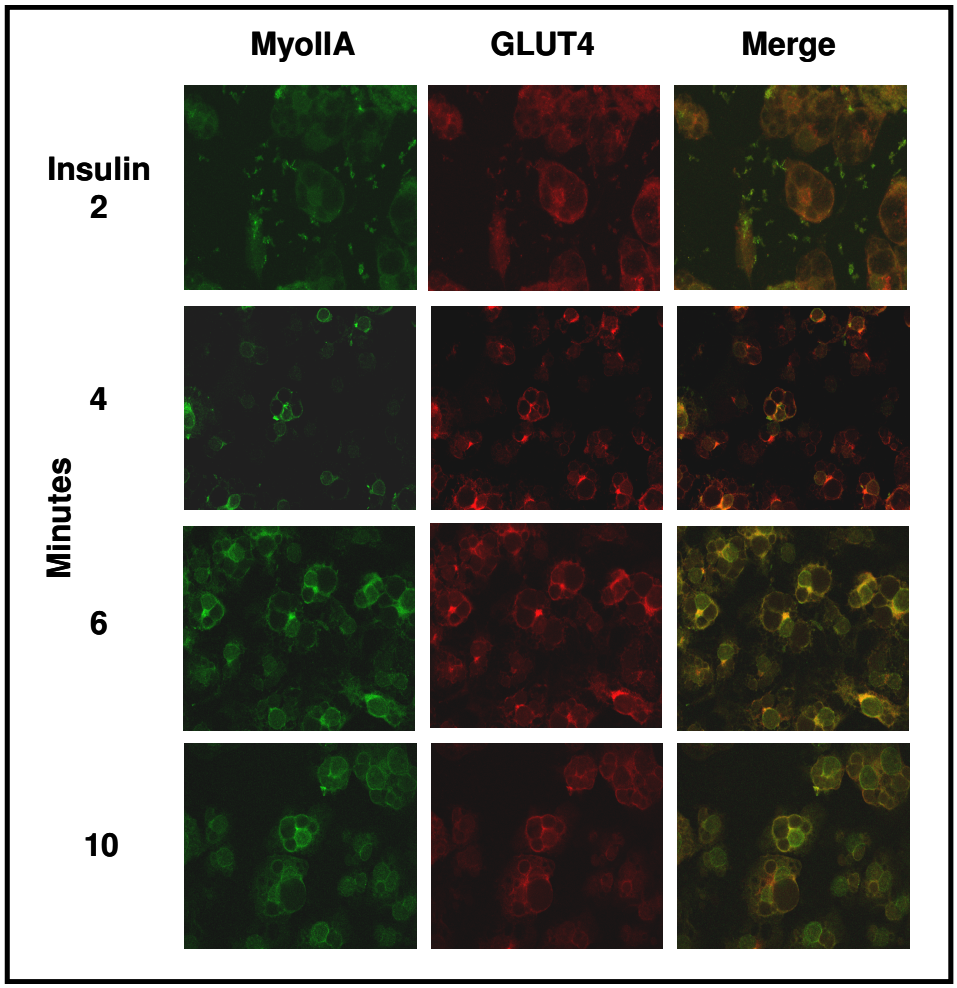

Figure 9. Treatment with blebbistatin impaired insulin stimulated recruitment of myosin IIA. 3T3-L1 adipocytes were incubated in the presence of blebbistatin and were treated according to the time course insulin stimulation protocol as described in materials and methods. Myosin IIA and GLUT4 were visualized by immunofluorescence using antibodies specific to myosin IIA and GLUT4. Myosin IIA and GLUT4 localization was analyzed in the basal condition and after stimulation with insulin at 2,4,6 and 10 minute time points using confocal microscopy. The results are representative images from three independent experiments.

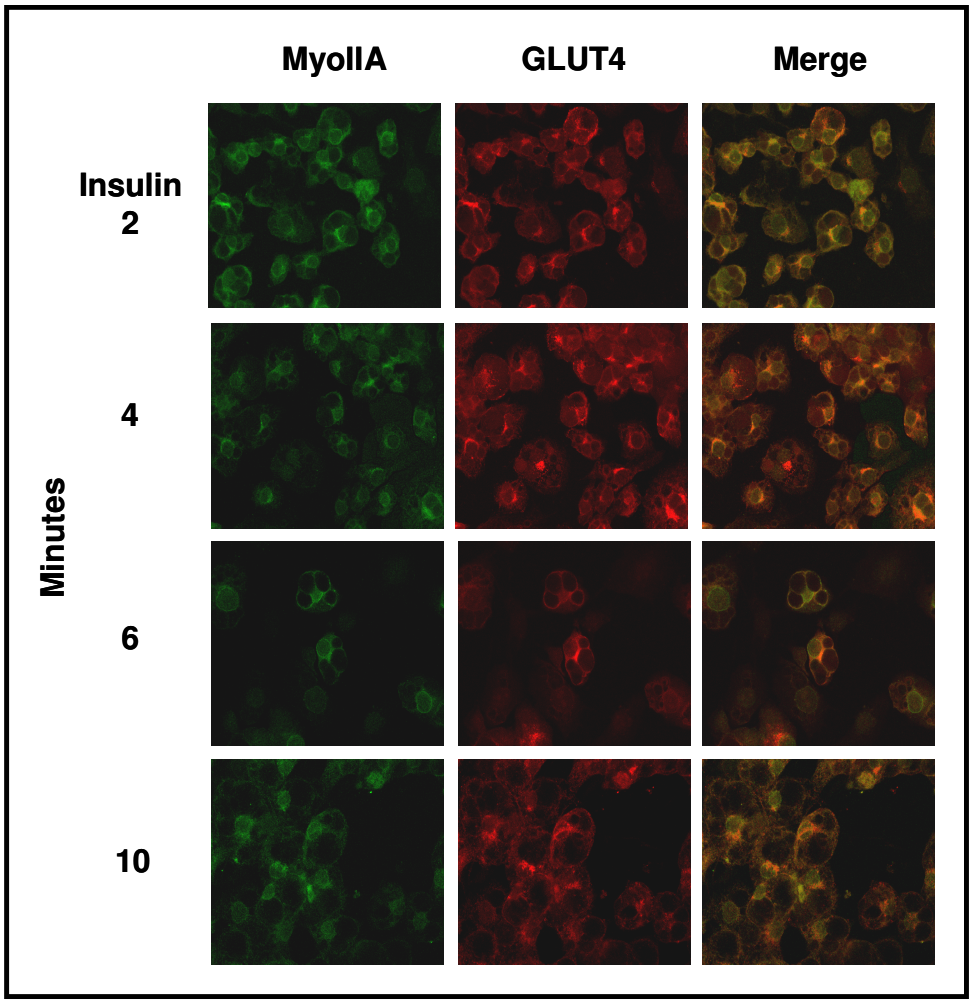

Figure 10. Treatment with ML-7 impaired insulin stimulated myosin IIA recruitment. 3T3-L1 adipocytes were incubated in the presence of ML-7 and were treated according to the time course insulin stimulation protocol as described in materials and methods. Myosin IIA and GLUT4 were visualized by immunofluorescence using antibodies specific to myosin IIA and GLUT4. Myosin IIA and GLUT4 co-localization was analyzed in the basal condition and after stimulation with insulin at 2,4,6 and 10 minute time points using confocal microscopy. The results are representative images from three independent experiments.

Temporal assays were performed and confocal microscopy was used to identify patterns of co-localization of myosin IIA and GLUT4 during a time course insulin stimulation assay. The localization of myosin II and GLUT4 were determined at 0, 2, 4, 6 and 10 minutes after insulin stimulation using confocal microscopy.

I observed that myosin IIA remained localized at the perinuclear region in cells that had been treated with ML-7 (Fig. 10; Myosin IIA panel). These patterns are consistent with the basal controls seen in Fig. 8; A - C. Inhibition of myosin IIA activity did not affect the translocation of GLUT4 containing vesicles to the plasma membrane (Fig. 10; GLUT4 panel). In the merged images of the ML-7 treatment there is significantly less co-localization of myosin IIA and GLUT4 at ten minutes due to the inhibition of myosin II activation. This is indicated by the low levels of yellow coloration observed at the plasma membrane. This indicates that myosin IIA and GLUT4 are not co-localized at the plasma membrane (Fig. 10; 10 minutes).

In this investigation I have revealed that stimulating 3T3-L1 adipocytes with insulin resulted in the recruitment of myosin IIA to the plasma membrane from the perinuclear region. I have shown that chemical inhibition of the activation of myosin II resulted in a disruption of the dynamic recruitment of myosin IIA to the plasma membrane and this inhibition resulted in impaired insulin stimulated glucose uptake [1] (Fig. 3). Translocation of GLUT4 to the plasma membrane was shown to be independent of myosin II activity (Fig.; 6 -

10). Inhibiting myosin II activity by inhibiting the phosphorylation of the RLC resulted in myosin IIA remaining at the perinuclear region rather than translocating to the plasma membrane. These results indicate that the recruitment of myosin IIA to the plasma membrane is regulated via myosin light chain kinase. It is important to directly determine if the phosphorylated state of the RLC of myosin IIA is being regulated by insulin.

Myosin IIA is activated during insulin stimulated glucose uptake

My results have thus far identified that the myosin II isoforms IIA and IIB do exhibit distinct patterns of localization and recruitment during insulin stimulated glucose uptake in 3T3-L1 adipocytes. Since these patterns were identified it was important to identify which of the two myosin II isoforms is being activated by insulin. Myosin II motor activity and parallel filament assembly are regulated by the phosphorylation of residues localized on the RLC [5, 10]. Detection of the phosphorylated RLC was used to indicate which isoforms are active during insulin stimulated glucose uptake.

To determine if myosin IIA, myosin IIB or both, are activated during insulin stimulation, cells were incubated in the presence of DMSO, DMSO and insulin or insulin and either blebbistatin or ML-7. The cells were lysed and the lysates were collected for use in immunoprecipitation assays. Antibodies specific to myosin IIA, myosin IIB, RLC or the phosphorylated RLC were used to immunoprecipitate their respective antigen from the collected lysates. All of the immunoprecipitates

were also immunoblotted and probed with each of the antibodies used in this investigation as a control to verify immunoprecipitation and to identify any cross reactivity.

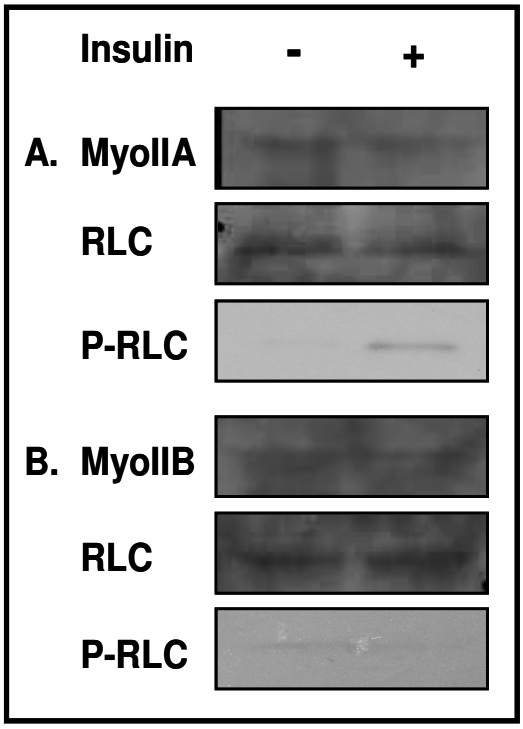

Figure 11. Effect of insulin stimulation on the activation of myosin IIA and myosin IIB. 3T3-L1 adipocytes were treated according to the insulin stimulation protocol as described in materials and methods. Cell lysates were immunoprecipitated with antibodies specific to A. myosin IIA or B. myosin IIB. Immunoprecipitates were resolved by SDS-PAGE and then probed with antibodies against myosin IIA, myosin IIB, RLC or Phosphorylated-RLC. The results are representative of three independent experiments.

I observed that the RLC associated with myosin IIA was phosphorylated in cells stimulated with insulin. In the presence of insulin myosin IIA was shown to have a significant increase in the amount of phosphorylated RLC as compared to the basal controls (Fig. 11).

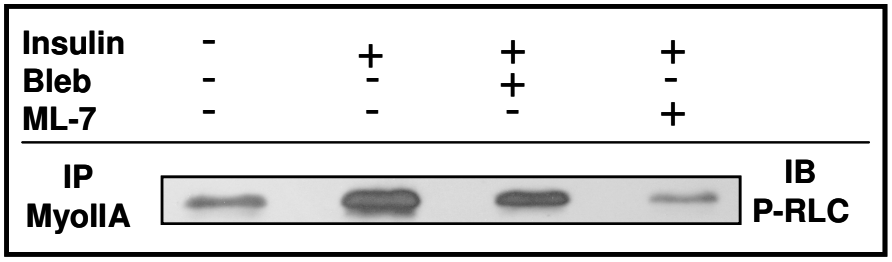

Figure 12. Inhibiting the activity of myosin II affects the phosphorylation status of the RLC.. 3T3-L1 adipocytes were pre-treated with 100 µM blebbistatin or 10 µM ML-7 for four hours and then treated according to the insulin stimulation protocol as described in materials and methods. Cell lysates were then immunoprecipitated with antibodies specific to myosin IIA. Immunoprecipitates were resolved by SDS-PAGE and then probed with antibodies against phosphorylated-RLC. The results are representative of three independent experiments.

In 3T3-L1 adipocytes treated with blebbistatin plus insulin the amount of phosphorylated RLC observed in myosin IIA was relatively unaffected compared to the insulin only control (Fig. 12). A decrease in the amount of phosphorylated RLC was observed in myosin IIA in cells treated with ML-7 plus insulin compared to the insulin only control (Fig. 12).

I have identified that myosin IIA and myosin IIB are both expressed in 3T3-L1 adipocytes and that these isoforms exhibit distinct patterns of localization following those observed in other cell types where myosin IIA localizes to dynamic regions of cells / tissues. I have also shown that myosin IIA is actively recruited from the perinuclear region to areas at the plasma membrane

containing GLUT4 in 3T3-L1 adipocytes (Fig.; 7-9). The results from the immunoprecipitation assays indicate that myosin IIA is activated during insulin stimulation by phosphorylation of the RLC. It is shown that by inhibiting the interaction of myosin II with actin with blebbistatin results in no significant change in the phosphorylation status of myosin IIA RLC. Inhibiting the activation of myosin II in a MLCK dependent manner results in an observed decrease of phosphorylated RLC detected in myosin IIA. These results demonstrate that the activation and subsequent recruitment of myosin IIA to the plasma membrane in 3T3-L1 adipocytes is regulated by insulin. These results provide support for my hypothesis that the localization and activation of myosin II isoforms are regulated by insulin during insulin stimulated glucose uptake in 3T3-L1 adipocytes.

CHAPTER IV

DISCUSSION

The purpose of this study was to test for a potential role of myosin II in insulin stimulated glucose uptake. This was dond by determining the localization patterns of myosin II isoforms in 3T3-L1 adipocytes during insulin stimulation and to determine whether the recruitment of myosin II was regulated by insulin. I have shown that in 3T3-L1 adipocytes myosin IIA is localized to the perinuclear region in the basal state and is recruited to the plasma membrane after stimulation with insulin (Fig 6; A and B; Fig 7; A and D). Myosin IIB was shown to remain localized at the plasma membrane in 3T3-L1 adipocytes in the basal state and throughout the process of insulin stimulated glucose uptake (Fig. 6; C and D). The results presented in this study indicate that myosin IIA may be recruited in an insulin dependent manner to the plasma membrane to initiate the process of reorganizing the cortical layer of actin found in 3T3-L1 adipocytes.

This report identifies the same recruitment pattern of cytoplasmic myosin IIA to dynamic areas as previously identified in gastric parietal cells lamellipodial extensions via histamine stimulation [37]. The recruitment patterns of myosin IIA identified in various cell types including 3T3-L1 adipocytes put myosin IIA at areas in which cytoskeletal components are being altered or rearranged.

I hypothesized that myosin IIA is dynamically or actively recruited to the plasma membrane. M y data indicates that by treating cells with the myosin II specific inhibitor blebbistatin, myosin IIA remained localized to the perinuclear region in adipocytes while the recruitment of GLUT4 to the plasma membrane was unaffected (Fig. 9). Blebbistatin blocks the ATPase activity of myosin II and its interaction with actin and results in active myosin IIA remaining localized at the perinuclear region of adipocytes. Inhibition of myosin II activity associated with actin resulted in a significant impairment of insulin stimulated glucose uptake as reported in my previous study [1]. By revealing that the inhibition of myosin II ATPase activity affects the recruitment of actin-associated myosin IIA to the plasma membrane, it was important to identify if only active, phosphorylated myosin IIA was recruited to the plasma membrane upon insulin stimulation. By treating cells with the MLCK specific inhibitor ML-7, it was shown that the recruitment of myosin IIA to the plasma membrane was greatly impaired although the level of GLUT4 vesicles recruited to the plasma membrane was not (Fig.9 and Fig. 10). This indicates that only active myosin IIA that is associated with actin is recruited to the plasma membrane and that the recruitment of GLUT4 is independent of the activity of myosin IIA. These results taken together suggest that inhibiting the recruitment of active myosin IIA to the plasma membrane or that inhibiting the association of myosin IIA with actin impairs glucose uptake.

Investigation of the patterns of co-localization of myosin IIA and GLUT4 in 3T3-L1 adipocytes revealed that in the basal state, myosin IIA and GLUT4 were

found to be co-localized at the perinuclear region (Fig. 7; A-C). Upon insulin stimulation, active myosin IIA was recruited to specific regions where GLUT4 had translocated to at the plasma membrane in adipocytes (Fig. 7; D-F). These results are the first to identify that myosin IIA co-localizes with GLUT4 to specific regions at the plasma membrane in 3T3-L1 adipocytes. It was shown that by inhibiting both the interaction of myosin II with actin and also by inhibiting the phosphorylation of the RLC, myosin IIA remained localized at the perinuclear region of 3T3-L1 adipocytes. In these treatments GLUT4 was shown to translocate to the plasma membrane with no co-localization of myosin IIA at the plasma membrane (Fig. 7). This result indicates that both the interaction of myosin IIA with actin and the phosphorylation of the RLC of myosin IIA is required for its recruitment to the plasma membrane during insulin stimulated glucose uptake.

The results in this study have identified that the recruitment of myosin IIA is independent of GLUT4 translocation to the plasma membrane during insulin stimulation. With this pattern revealed it was important to identify when myosin IIA is recruited to the plasma membrane in relation to the recruitment of GLUT4 during insulin stimulation. A temporal relationship of the recruitment pattern of myosin IIA and GLUT4 was identified in 3T3-L1 adipocytes.GLUT4 translocated to the plasma membrane prior to myosin IIA after insulin stimulation (Fig. 8). Results from my previous study show that by treating adipocytes with blebbistatin insulin stimulated glucose uptake was impaired in 3T3-L1 adipocytes (Fig. 3 and

Fig. 8) [1]. The results presented in this report identify that GLUT4 levels at the plasma membrane increase up to the 10 minute time point although the recruitment of active myosin IIA to the plasma membrane was impaired

Since myosin IIA is actively recruited to the plasma membrane it was important to identify if this recruitment was dependent upon the activation of myosin IIA. Previous studies have shown that myosin IIA can be recruited to areas where distinct cellular processes are occurring without being activated via phosphorylation of the RLCs as seen in the recruitment of inactive myosin IIA to the furrow of dividing HeLa cells [38]. Activation of myosin IIA was inhibited using ML-7 in that study. These studies resulted in myosin IIA remaining localized at the perinuclear region during insulin stimulation since non-active myosin II cannot interact with actin (Fig. 9). The levels of GLUT4 recruited to the plasma membrane were shown to be unaffected by inhibiting the activation of myosin IIA (Fig. 9). These results indicate that the activation of myosin IIA is also required for its recruitment to the plasma membrane. Thus far I have identified that the recruitment of myosin IIA is independent of the translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes. I have also revealed that myosin IIA translocates and co-localizes to specific areas at the plasma membrane where GLUT4 is localized. Furthermore, I have identified that by inhibiting both myosin IIA activity and its association with actin results in myosin IIA remaining localized at the perinuclear region in 3T3-L1 adipocytes. These results further support the

idea that the recruitment of myosin IIA is dependent upon its activity and association with actin in 3T3-L1 adipocytes.

Since the recruitment of myosin IIA is dependent upon its activity and the recruitment of active myosin IIA is dependent upon its association with actin; it was important to identify the activation patterns of myosin II during insulin stimulated glucose uptake. I identified that the activation of myosin IIA is regulated by insulin signaling. Activation was determined by assaying the P- RLC of myosin IIA after insulin stimulation. RLC phosphorylation was increased only in the presence of insulin (Fig. 11 and Fig. 12). Treating cells with the chemical inhibitor blebbistatin resulted in levels of phosphorylated RLC comparable to that seen in the insulin controls (Fig. 12). These results show that inhibiting the ATPase activity of myosin IIA pharmacologically does not inhibit the activation of myosin IIA but does inhibit its active recruitment to the plasma membrane in 3T3-L1 adipocytes. I have identified that by inhibiting of the activation of myosin IIA by inhibiting MLCK results in a decrease of phosphorylated RLC (Fig.12). These results further support the results from my confocal microscopy studies indicating that the recruitment of myosin IIA to the plasma membrane is dependent upon its activation.

The results from this study are the first to identify that myosin IIA activity and association with actin are dependent on insulin. Continuation of these studies is important to reveal the details regulating this interaction involving myosin II, GLUT4 and actin. Revealing the underlying interactions and

mechanisms affecting insulin stimulated glucose uptake may provide important information that could be used to optimize current treatments or develop new treatments for type II diabetes.

LITERATURE CITED

[1] P.A. Steimle, F.K. Fulcher, Y.M. Patel, A novel role for myosin II in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Biochem Biophys Res Commun 2005; 331:1560-5.

[2] R.T. Watson, M. Kanzaki, J.E. Pessin, Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. Endocr Rev. 2004; 25:177-204

[3] C. Pamela, R.A. Champe, D.R. Harvey, Lippincott's Illustrated Reviews: Biochemistry. Lippincott Williams & Wilkins; 3rd edition. 2004

[4] C. Taha and A. Klip, The insulin signaling pathway. J Membr Bio.1999; 169:1–12

[5] A.D. Kohn, S.A. Summers, M.J. Birnbaum and R.A. Roth, Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem.1996; 271:31372–31378

[6] A.W. Harmon, D.S. Paul and Y.M. Patel, MEK inhibitors impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Am. J Physio Endocrino Metab. 004; 287:E758–E766

[7] M. Kanzaki and J.E. Pessin, Insulin-stimulated GLUT4 translocation in adipocytes is dependent upon cortical actin remodeling. J Biol Chem.2001; 276:42436–42444

[8] T. Tsakiridis, P. Tong, B. Matthews, E. Tsiani, P.J. Bilan, A. Klip and G.P. Downey, Role of the actin cytoskeleton in insulin action. Microsc Res Tech.1999; 47:79–92

[9] A.Bose,Guilherme,S.I. Robida,S.M. Nicoloro,Q.L. Zhou ,Z.Y. Jiang, D.P.Pomerleau, Czech MP, Glucose transporter recycling in response to insulin is facilitated by myosin Myo1c. Nature. 2002; 420:821–824

[10] A.R. Bresnick, Molecular mechanisms of nonmuscle myosin-II regulation. Curr Opin Cell Biol.1999;11:26–33

[11] J. Kolega, Fluorescent analogues of myosin II for tracking the behavior of different myosin isoforms in living cells. J Cell Biochem.1998; 68:389–401

[12] J. Kolega, Cytoplasmic dynamics of myosin IIA and IIB: spatial ‘sorting’ of isoforms in locomoting cells. J Cell Sci.1998; 111:2085–2095

[13] M. Charron, J.Gorovits,L. Naira,J Skye,M & K Ranalletta,B. Ellen, Use of GLUT-4 Null Mice to study Skeletal Muscle Glucose Uptake. Clin and Exp Pharma and Phys.1999; 32:308-13

[14] J.S. Allingham, R. Smith,I Rayment. The structural basis of blebbistatin inhibition and specificity for myosin II. Nat Struct Mol Biol.2005; 4:378-9

[15] D.S. Paul, A.W. Harmon, C.P. Winston and Y.M. Patel, Calpain facilitates GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes. J Biochem. 2003; 376:625–632

[16] M. Kovacs , J.Toth,C.Hetenyi,A.C. Malnasi, J.R. Sellers. Mechanism of blebbistatin inhibition of myosin II.J Biol Chem. 2004; 34:35557-63

[17] B.Ramamurthy,C.M. Yengo,A.F. Straight, T.J. Mitchison,H.L. Sweeney. Kinetic mechanism of blebbistatin inhibition of nonmuscle myosin IIb.Biochemistry. 2004; 43:14832-9

[18] S.Shu,X Liu,E.D. Korn. Blebbistatin and blebbistatin-inactivated myosin II inhibit myosin II-independent processes in Dictyostelium.Proc Natl Acad Sci U S A. 2005; 102:1472-7

[19] Korn E.D. The discovery of unconventional myosins: serendipity or luck? J Biol Chem.2004;279:8517-25

[20] M. Guha, M. Zhou and Y. Wang .Cortical Actin Turnover during Cytokinesis Requires Myosin II**.** Current Biology. 2005; 15:732-736

[21] K. Murthy,P Wadsworth. Myosin-II-dependent localization and dynamics of F-actin during cytokinesis.Curr Biol. 2005; 15:724-31

[22] A.Yoneda,H.A. Multhaupt,J.R. Couchman.The Rho kinases I and II regulate different aspects of myosin II activity.J Cell Biol. 2005; 170:443-53

[23] D. Xia, J. T. Stull, K. E. Kamm Myosin phosphatase targeting subunit 1 affects cell migration by regulating myosin phosphorylation and actin assembly.Exp Cell Research.2005;304:506-517

[24] Y. Choi, H. Jeong, H. Rim Kim,S.S. Young,K. Cheonghwan, L. Wan, C.Han, H.L.Chae, Y.J. Jang. Implication of phosphorylation of the myosin II regulatory light chain in insulin-stimulated GLUT4 translocation in 3T3-F442A adipocytes. Exp Mol Med.2006; 38:180-189

[25] P.Maupin, C.L. Phillips, R.S. Adelstein, D.T. Pollard. Differential localization of myosin-II isozymes in human cultured cells and blood cells. J Cell Sci.1994, 107:3077-3090

[26] M.W**.**Rochilin, K. Itoh, R.S. Aldestein, P.C. Bridgman. Localization of myosin IIA and B isoforms in cultured neurons. J Cell Sci.1995; 108:3661-3670.

[27] J.Goldman, L. Zhong L, S.Q. Liu. Negative Regulation of Vascular Smooth Muscle Cell Migration by Blood Shear Stress. Am J Physiol Heart Circ Physiol. 2006 [Epub ahead of print]

[28] S.P.Srinivas , M.Satpathy , Y.Guo ,V. Anandan . Histamine-induced phosphorylation of the regulatory light chain of myosin II disrupts the barrier integrity of corneal endothelial cells. Invest Ophthalmol Vis Sci. 2006; 479:4011-8

[29] I.Krits, R.B. Wysolmerski, L.S.Holliday, B.S. Lee. Differential localization of myosin II isoforms in resting and activated osteoclasts. Calcif Tissue Int. 2002; 71:530-8

[30] T.Saitoh, S.Takemura , K. Ueda ,H. Hosoya,M. Nagayama ,H. Haga , K. Kawabata, A.Yamagishi, M.Takahashi. Differential localization of non-muscle myosin II isoforms and phosphorylated regulatory light chains in human MRC-5 fibroblasts. FEBS Lett. 2001; 509:365-9

[31] G. Eitzen, Actin remodeling to facilitate membrane fusion.Biochim. Biophys. Acta 2003; 641181.

[32] P. neco, D. Giner, S. Viniegra, R. Borges, A. Villarroel, L.M. Gutierrez, New roles of myosin II during vesicle transport and fusion in chromaffincells, J Biol Chem.2004:27450-57.

[33] X.Donglan *, T. James., K. E. Kamm. Myosin phosphatase targeting subunit 1 affects cell migration byregulating myosin phosphorylation and actin assembly. Experimental Cell Research. 2005; 304:506– 517

[34] K.E. Kamm, J.T. Stull, Dedicated myosin light chain kinases with diverse cellular functions, J Biol Chem.2001; 276:4527– 4530

[35] D.J. Hartshorne, M. Ito, F. Erdodi, Myosin light chain phosphatase:subunit composition, interactions and regulation, J Muscle Res Cell Motil. 1998; 19:325– 341

[36] D.J. Hartshorne, Myosin phosphatase: subunits and interactions, Acta Physiol Scand.1998; 164:483– 493

[37] R. Zhou, C. Watson, C. Fu, X. Yao, J.G. Forte, Myosin II is present in gastric parietal cells and required for lamellipodial dynamics associated with cell activation.Am. J Physiol Cell Physiol.2003; 119:C662-C673