

A novel role for myosin II in insulin-stimulated glucose uptake in 3T3-L1 adipocytes

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Abstract:

Insulin-stimulated glucose uptake requires the activation of several signaling pathways to mediate the translocation and fusion of GLUT4 vesicles from an intracellular pool to the plasma membrane. The studies presented here show that inhibition of myosin II activity impairs GLUT4-mediated glucose uptake but not GLUT4 translocation to the plasma membrane. We also show that adipocytes express both myosin IIA and IIB isoforms, and that myosin IIA is recruited to the plasma membrane upon insulin stimulation. Taken together, the data presented here represent the first demonstration that GLUT4-mediated glucose uptake is a myosin II-dependent process in adipocytes. Based on our findings, we hypothesize that myosin II is activated upon insulin stimulation and recruited to the cell cortex to facilitate GLUT4 fusion with the plasma membrane. The identification of myosin II as a key component of GLUT4-mediated glucose uptake represents an important advance in our understanding of the mechanisms regulating glucose homeostasis.

Keywords: Myosin II; Blebbistatin; Glucose uptake; Adipocytes

Article:

Glucose uptake is the rate-limiting step in glucose utilization in adipocytes. Insulin-stimulated glucose uptake requires the activation of several signaling pathways that stimulate the translocation and fusion of glucose transporter (GLUT4)-containing vesicles to the plasma membrane (reviewed in [1]). The binding of insulin to its receptor initiates a cascade of events involving phosphorylation of insulin receptor substrate (IRS) proteins and the subsequent activation of phosphatidylinositol-3 kinase (PI3 kinase) [2]. In turn, PI3 kinase activates downstream targets, such as the serine/threonine protein kinase Akt/PKB and atypical protein kinase C (aPKC), both of which are involved in the translocation of GLUT4 vesicles to the plasma membrane and glucose uptake into the cell [3] and [4]. Insulin also stimulates glucose uptake via two PI3 kinase-independent pathways: the mitogen activated protein kinase (MAPK) pathway and the Cbl pathway [1], [3] and [5].

Previous studies have demonstrated that insulin-stimulated GLUT4 translocation and membrane fusion in adipocytes requires cortical actin reorganization [6], [7] and [8]. Mature adipocytes do not have an extensive array of stress fibers but instead have a layer of cortical actin filaments (F-actin) that lines the inner surface of the plasma membrane. In adipocytes, the actin cytoskeleton may function as a “track” on which GLUT4 vesicles are translocated from intracellular pools to the plasma membrane [7] and [9]. In addition, actin filaments localized at the cell cortex can act as a barrier that must be “loosened/relaxed” in order for vesicles to fuse with the plasma membrane [10], [11] and [12]. While a role for actin reorganization in GLUT4 trafficking has been established, relatively little is known about the contractile forces facilitating actin reorganization that can lead to localized remodeling of the cell cortex required for vesicle fusion at the plasma membrane. Myosin II, also called conventional myosin, plays an important role in a wide variety of processes in eukaryotic cells by supporting ATP-dependent contraction of actin filaments. In skeletal muscle cells, myosin II bipolar filaments are arranged into stable, ordered arrays between actin filaments and the mechanisms by which myosin mediates myofibril contraction have been well characterized [13]. In comparison, mammalian nonmuscle cells express myosin II isoforms that can undergo dramatic changes in localization and activation as a part of various cellular events [14]. A variety of studies in lower eukaryotes such as *Dictyostelium*, *Acanthamoeba*, and

Saccharomyces cerevisiae have revealed that myosin II, in a nonmuscle cell context, plays a central role in vital cell processes such as cytokinesis, cell locomotion, maintenance of cell cortical tension, and cell surface receptor capping [14] and [15]. Recent studies in mammalian nonmuscle cell lines, such as 3T3 fibroblasts and COS-1 cells, indicate that myosin II plays an important role in regulating the cytoskeletal reorganization required for vesicle fusion with the plasma membrane [16] and [17].

The experiments described in this report are the first to explore the role of myosin II in regulating the dynamic cellular processes driving insulin-stimulated glucose uptake in adipocytes. More specifically, we have found that treatment of 3T3-L1 adipocytes with the myosin II-specific inhibitor blebbistatin significantly impairs insulin-stimulated glucose uptake, but does not affect GLUT4 translocation to the plasma membrane. Blebbistatin is a cell permeable and highly specific small molecule inhibitor of nonmuscle myosin II that does not alter the activities of myosins I, V, or X [18], [19], [20], [21] and [22]. The studies described here also show for the first time that adipocytes express both myosin IIA and IIB isoforms, and that in unstimulated adipocytes myosin IIA is localized primarily in the perinuclear region of the cell while myosin IIB is highly enriched at the cell cortex. Further studies reveal that insulin stimulation of adipocytes leads to myosin IIA relocalization to the cell cortex. Collectively, our results are the first to reveal that myosin II plays a critical role in mediating insulin-stimulated glucose uptake in 3T3-L1 adipocytes, possibly via a mechanism that facilitates GLUT4 vesicle fusion at the plasma membrane. In a broader context, the identification of myosin II as a major component of GLUT4-mediated glucose uptake provides important new insight into the mechanisms by which proper glucose homeostasis is maintained in cells.

Materials and methods

Materials. Tissue culture reagents were obtained from Gibco (Grand Island, NY). Insulin was purchased from Roche Diagnostics (Indianapolis, IN). Dexamethasone, 3-isobutyl-1-methyl-xanthine, and myosin IIA antibody were from Sigma (St. Louis, MO). Blebbistatin was purchased from Calbiochem (San Diego, CA). Phospho-Akt (Ser⁴⁷³), phospho-Akt (Thr³⁰⁸), and Akt antibodies were from Cell Signaling Technology (Beverly, MA). GLUT4 antibody (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The myosin IIB antibody and Texas red-phalloidin were obtained from Covance (Berkeley, CA). Alexa Fluor 594 donkey anti-goat IgG and goat anti-rabbit IgG were from Molecular Probes (Eugene, OR). The enhanced chemiluminescence (ECL) detection kit and horseradish peroxidase-conjugated secondary antibodies were from Amersham Bioscience (Piscataway, NJ).

Glucose uptake assay. 3T3-L1 pre-adipocytes were induced to differentiate as described previously [23]. Glucose uptake assays were performed on fully differentiated 3T3-L1 adipocytes. Adipocytes were serum starved for 4 h in the presence of 0.1% DMSO (vehicle) or 100 μ M blebbistatin. Adipocytes were then washed twice with 37 °C Krebs-Ringer Phosphate (KRP) buffer (pH 7.4) containing 128 mM NaCl, 4.7 mM KCl, 1.65 mM CaCl₂, 2.5 mM MgSO₄, and 5 mM Na₂HPO₄, and then placed in KRP buffer containing vehicle or 100 μ M blebbistatin. Adipocytes were either untreated (basal) or treated with insulin (100 or 1 nM) for 10 min, followed by the addition of [1-¹⁴C]2-deoxy-d-glucose (0.1 μ Ci/well) (NEN) and 5 mM glucose for an additional 10 min at 37 °C. Cells were then washed three times with phosphate-buffered saline (PBS) and solubilized in 0.5 M NaOH and 0.1% SDS. Samples were assayed for [¹⁴C]2-deoxy-d-glucose uptake as disintegrations per min per mg protein.

Immunoblot analysis. 3T3-L1 adipocytes were lysed in 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 Na₃VO₄, 1 mM NaPP_i, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin, 5 mg/ml leupeptin, and 5 mg/ml pepstatin [7]. Lysates were incubated for 20 min at 4 °C and then centrifuged at 6000g for 20 min at 4 °C. The resulting supernatants were incubated for 5 min at 95 °C in Laemmli sample buffer [24] and then separated by 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and analyzed by immunoblotting as previously described [23].

Immunofluorescence. Fully differentiated adipocytes grown on coverslips were serum starved for 4 h in the presence of 0.1% DMSO or 100 μ M blebbistatin and treated according to the glucose uptake protocol (omitting [14 C]2-DOG). Cells were then fixed with 2% buffered paraformaldehyde, permeabilized in 0.25% Triton X-100 (Sigma–Aldrich) for 5 min at 4 $^{\circ}$ C, and incubated with anti-Glut-4 antibody, anti-myosin IIA antibody, or anti-myosin IIB antibody. The slides were then incubated with an anti-goat or anti-rabbit Alexaflour 594 labeled antibody (Molecular Probes, Eugene, OR) depending on the origin of primary antibody (see Materials). Actin was visualized using Texas red–phalloidin. Plasma lawn assays were performed as described previously [7] and [23]. Slides were viewed using an Olympus IX81 Motorized Inverted Confocal Microscope and FLUOVIEW FV500 software. The relative intensity of cortical immunofluorescence was quantified using Image-Pro Plus software (Silver Spring, MD) [7].

Results

Myosin II is required for GLUT4-mediated glucose uptake

In mature adipocytes, GLUT4-containing vesicles translocate, dock, and then fuse to the plasma membrane in response to insulin stimulation; these events ultimately drive insulin-stimulated glucose uptake in these cells. Previous studies have revealed that myosin II plays an important role in vesicle trafficking in various cellular contexts [16], [17], [25] and [26]. In the experiments described here, we examined the role of myosin II in insulin-stimulated glucose uptake in 3T3-L1 adipocytes using the myosin II-specific inhibitor, blebbistatin. To this end, fully differentiated adipocytes were serum starved for 4 h in the presence or absence of blebbistatin (100 μ M) and then glucose uptake assays were performed using [14 C]2-deoxy-d-glucose. In the absence of blebbistatin, insulin (100 nM) stimulated a dramatic increase in glucose uptake over basal levels (Fig. 1A). In contrast, treatment of cells with blebbistatin inhibited insulin-stimulated glucose uptake by approximately 66% compared with the vehicle control, suggesting that myosin II activity is a necessary component of this process (Fig. 1A). While insulin stimulates a dramatic increase in glucose uptake primarily via the GLUT4 transporter, there is also a slight increase in the translocation of GLUT1 to the plasma membrane [27]. To determine if the effect of blebbistatin treatment on glucose uptake is attributable to inhibition of GLUT4 or GLUT1, we treated adipocytes with 1.0 nM insulin; this concentration of insulin stimulates GLUT4, but not GLUT1, recruitment to the plasma membrane [27]. Under these conditions, blebbistatin (100 μ M) blocked glucose uptake by approximately 70% compared with the vehicle-treated control (Fig. 1B). Taken together, these data are the first to reveal that myosin II plays a critical role in the process of GLUT4-mediated glucose uptake in response to insulin stimulation in adipocytes.

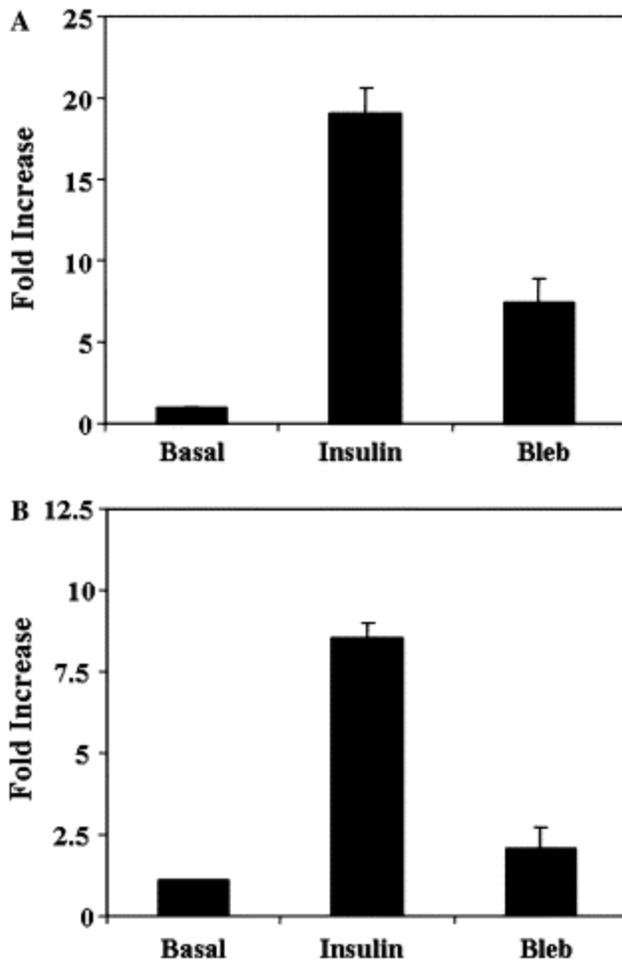


Fig. 1. Inhibition of myosin II impairs insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Adipocytes were serum starved in the presence of vehicle (0.1% DMSO) or 100 μ M blebbistatin, and then stimulated with either (A) 100 nM insulin or (B) 1.0 nM insulin for 10 min and assayed for [14 C]2-deoxy-d-glucose uptake in the presence of vehicle or 100 μ M blebbistatin. Glucose uptake was calculated as disintegrations per mg protein and expressed as percent of the vehicle control. Results are means \pm SEM of three independent experiments.

Inhibition of myosin II with blebbistatin does not block the insulin-induced phosphorylation of Akt, a distal event in the insulin signaling pathway

To elucidate the mechanism by which insulin-stimulated glucose uptake is inhibited by blebbistatin, we examined the phosphorylation of Akt, a distal event in the insulin-stimulated PI3 kinase signaling pathway. Insulin stimulates the phosphorylation of Akt at two regulatory sites, Thr³⁰⁸ and Ser⁴⁷³; phosphorylation of both residues is required for complete activation of Akt. Treatment of cells with 100 μ M blebbistatin had no effect on insulin-induced phosphorylation of Akt residues Thr³⁰⁸ (data not shown) and Ser⁴⁷³, and did not affect the overall levels of Akt protein in the cells (Fig. 2). Moreover, blebbistatin did not affect the cellular levels of actin (data not shown). Taken together, it appears that the well-characterized inhibition of myosin II by blebbistatin [19], [20] and [21] is responsible for the inhibition of glucose uptake observed in the previous experiments (Fig. 1), and is not due to nonspecific interference with insulin-stimulated activation of the PI3 kinase signaling pathway or is the result of a toxic effect on the cells.

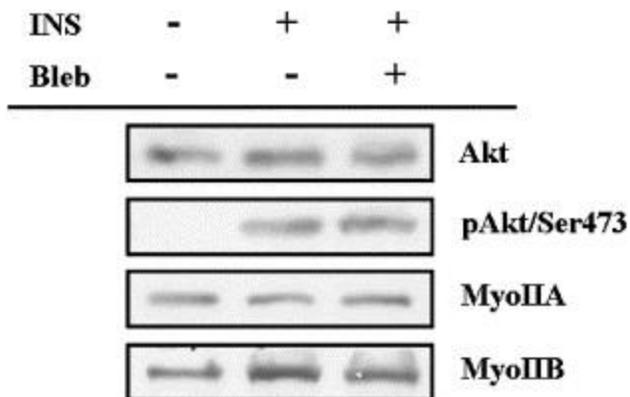


Fig. 2. Effect of blebbistatin on Akt phosphorylation. 3T3-L1 adipocytes were pre-treated with 0.1% DMSO vehicle or 100 μ M blebbistatin and were then left untreated (basal) or stimulated with 100 nM insulin for 10 min. Cell lysates were analyzed by SDS-PAGE, blotted to membrane, and then probed with antibodies against unphosphorylated Akt, phosphorylated Akt (Ser⁴⁷³ Akt), myosin IIA, and myosin IIB, as indicated. The results are representative of three independent experiments.

Insulin-stimulated GLUT4 translocation to the plasma membrane does not require myosin II activity

Insulin-stimulated glucose uptake requires the translocation of GLUT4-containing vesicles to the plasma membrane. Since inhibition of myosin II activity significantly reduced glucose uptake, we were interested in determining whether myosin II plays a role in insulin-stimulated GLUT4 translocation. To explore this possibility, the effect of blebbistatin treatment on the localization of GLUT4-containing vesicles was monitored by immunofluorescence confocal microscopy of unstimulated and insulin-stimulated adipocytes [14]. In untreated (basal level) cells, GLUT4 vesicles were localized primarily to the perinuclear region (Fig. 3, top panel). However, upon insulin stimulation, there was a marked increase in GLUT4 localization at the plasma membrane, as demonstrated by increased immunofluorescent staining at the cell membrane (Fig. 3, top panel). Blebbistatin treatment did not inhibit insulin-stimulated GLUT4 recruitment to the plasma membrane (Fig. 3, top panel), suggesting that myosin II activity is not involved in the translocation process.

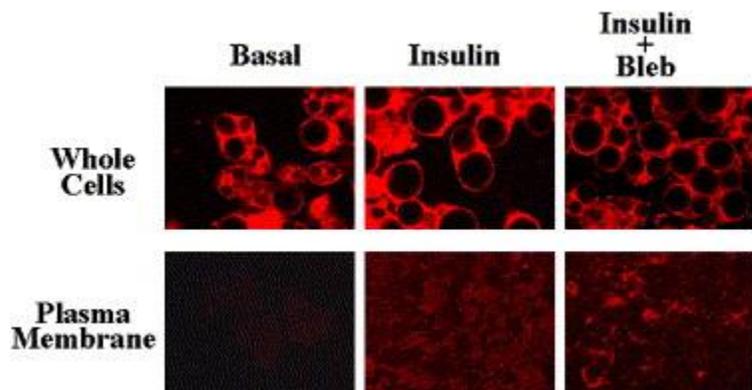


Fig. 3. Insulin-stimulated GLUT4 translocation was not affected by treatment with blebbistatin. Adipocytes were treated according to the glucose uptake protocol as described in Materials and methods, and GLUT4 protein was visualized by immunofluorescence using a GLUT4-specific antibody. GLUT4 localization 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 μ M blebbistatin. The results are representative images from three independent experiments.

In complementary studies, plasma lawn assays were performed to confirm quantitatively that inhibition of myosin II activity by blebbistatin did not alter insulin-stimulated GLUT4 translocation to the plasma membrane. Under basal conditions, only a low level of GLUT4 staining was detectable in plasma membrane sheets (Fig. 3,

bottom panel). Upon insulin stimulation, GLUT4 localization to plasma membrane sheets increased approximately 3-fold (Fig. 3, bottom panel). These results, as well as those described for the whole cell microscopy experiments described above, are consistent with numerous reports establishing that GLUT4 translocation to the plasma membrane occurs in response to insulin stimulation of adipocytes [1]. In accordance with our initial confocal studies (Fig. 3, top panel), we found that treatment of adipocytes with blebbistatin did not affect insulin-stimulated increases (2.5-fold) in GLUT4 protein to the plasma membrane (Fig. 3, bottom panel). These results indicate that while myosin II activity is required for insulin-stimulated glucose uptake in adipocytes, it does not appear that myosin II participates in GLUT4 vesicle translocation to the plasma membrane.

Myosin II isoforms exhibit distinct localization patterns in insulin-stimulated adipocytes

Previous studies have revealed distinctive patterns of localization for the two isoforms of myosin II depending on cell type and cellular context. Using confocal microscopy, we observed that under basal conditions, myosin IIA is localized primarily to the perinuclear region in adipocytes but is dynamically recruited to the plasma membrane upon insulin stimulation (Fig. 4). In contrast, myosin IIB is localized to the cell cortex, and this pattern of localization was not altered upon insulin stimulation (Fig. 4). To determine whether myosin II activity is required for its recruitment to the cell cortex, we treated adipocytes with blebbistatin and then stimulated with insulin. As shown in Fig. 4, myosin IIA was not recruited to the cell cortex in blebbistatin-treated cells, suggesting that myosin IIA activity is required for insulin-stimulated recruitment to the cell cortex. By comparison, the cortical localization of myosin IIB was unaffected by blebbistatin treatment. It is also important to note that the cellular levels of the myosin IIA and myosin IIB proteins did not change upon insulin stimulation of adipocytes (Fig. 2). Collectively, these findings indicate that the distinctive localization patterns displayed by the two isoforms of myosin II may reflect distinct functional roles in regulating vesicle trafficking and insulin-stimulated glucose uptake.

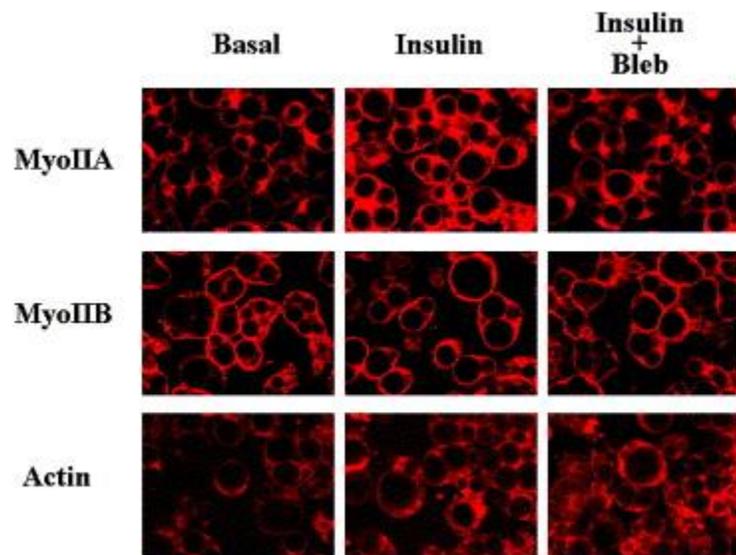


Fig. 4. The effect of insulin stimulation on myosin II localization in adipocytes. 3T3-L1 adipocytes were treated according to the glucose uptake protocol as described in Materials and methods and the myosin II isoforms were visualized by immunofluorescence using either a myosin IIA or myosin IIB-specific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

Discussion

Previous studies have revealed that the actin cytoskeleton plays a critical role in mediating GLUT4 vesicle trafficking in response to insulin stimulation of adipocytes. While the dynamic reorganization of actin filaments

has been shown to drive GLUT4 vesicle translocation to the cell cortex, the role of cortical actin in the fusion of GLUT4 vesicles with the plasma membrane is not well defined. In a nonmuscle cell context, members of the myosin family have been shown to shuttle cargo (vesicles) along cytoplasmic F-actin “tracks” [7], [9] and [28], and to mediate localized reorganization of actin filaments during processes such as cell migration and cytokinesis [14], [15] and [29]. Myosin II-mediated contraction of the actin cytoskeleton can also lead to localized remodeling of the cell cortex that may be required for vesicle fusion with the plasma membrane [12], [16] and [26]. The experiments presented here are the first to explore the role of myosin II in GLUT4-mediated glucose uptake and have identified myosin II as a novel component of the insulin signaling pathways involved in glucose homeostasis.

The findings presented here are the first to reveal that myosin II activity is involved in insulin-stimulated glucose uptake. We show that inhibition of myosin II activity by blebbistatin significantly reduced GLUT4-mediated glucose uptake in 3T3-L1 adipocytes, but did not affect GLUT4 vesicle translocation to the plasma membrane (Fig. 1 and Fig. 3). We further demonstrate that this inhibition of GLUT4-mediated glucose uptake did not interfere with the activation of Akt, a distal event in the insulin signaling pathway (Fig. 2). Taken together, these findings suggest that myosin II-mediated contraction of actin filaments participates in GLUT4-mediated glucose uptake at a terminal stage in GLUT4 trafficking, and thus indicates that myosin II may play a role in facilitating the docking and/or fusion of GLUT4 vesicles with the plasma membrane. Our findings are particularly significant since they are the first to demonstrate that the contraction of actin filaments by myosin II, in addition to actin reorganization (assembly/disassembly), is necessary for insulin-stimulated glucose uptake. By extension, the results presented here suggest that there may be two distinct mechanisms by which changes in the cytoskeleton (i.e., actin reorganization and myosin II-mediated contraction of actin filaments) contribute to the regulation of glucose homeostasis in adipocytes.

While inhibition of myosin II activity by blebbistatin treatment blocked GLUT4-mediated glucose uptake, it is still unclear which myosin II isoform is involved in this process. Our studies show that 3T3-L1 adipocytes express both myosin IIA and IIB isoforms, and that these isoforms exhibit distinct localization patterns and responses to insulin stimulation. Myosin IIA is localized to the perinuclear region under basal conditions and recruited to the plasma membrane upon insulin stimulation. In contrast, myosin IIB remains localized to the cell cortex in the presence or absence of insulin stimulation. These results are similar to those reported in a recent study showing that histamine stimulation of gastric parietal cells leads to the dynamic recruitment of the myosin IIA isoform to lamellipodial extensions from a diffuse cytoplasmic distribution [26]. The differential localization of the myosin IIA and IIB isoforms has also been reported for various cell types and contexts [14], [17], [26], [30], [31], [32] and [33].

Our studies showing that myosin IIA is recruited to the plasma membrane upon insulin stimulation of adipocytes may indicate that this isoform is responsible for cortical actin restructuring required for GLUT4 vesicle fusion with the plasma membrane. It is interesting to note that treatment of cells with blebbistatin prevented myosin IIA recruitment to the plasma membrane, suggesting that only active myosin IIA translocates to the cell cortex. In contrast, inhibition of myosin II did not prevent translocation of the GLUT4 vesicles, but did inhibit GLUT4-mediated glucose uptake. One possible explanation for these findings is that insulin signaling activates myosin IIA and promotes its recruitment to the plasma membrane to facilitate GLUT4 vesicle fusion. A previous study has reported that myosin IIA can bind to vesicles [34]; thus, it is tempting to speculate that active myosin IIA translocates with GLUT4 vesicles to the plasma membrane where it functions to facilitate vesicle fusion by altering cortical actin structure at distinct zones. Studies are in progress to further define the function of each myosin II isoform in facilitating insulin-stimulated glucose uptake in adipocytes. In summary, our studies have revealed a novel function for myosin II in facilitating insulin-stimulated glucose uptake in 3T3-L1 adipocytes. We also show that myosin II activity is important for mediating distal events in insulin signaling and may play a central role in facilitating GLUT4 vesicle fusion with the plasma membrane. Our results, along with those of others, indicate that myosin II may play a general role in vesicle trafficking in a variety of cellular contexts [16], [17], [25], [26], [34], [35] and [36]. The results presented here add to a rapidly

growing list of factors involved in promoting GLUT4-mediated glucose uptake and thus provide further insight into the cellular basis of impaired insulin sensitivity.

Acknowledgments

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