Disruption of Microtubules Ablates the Specificity of Insulin Signaling to GLUT4 Translocation in 3T3-L1 Adipocytes*

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Although the cytoskeletal network is important for insulin-induced glucose uptake, several studies have assessed the effects of microtubule disruption on glucose transport with divergent results. Here, we investigated the effects of microtubule-depolymerizing reagent, nocodazole and colchicine, on GLUT4 translocation in 3T3-L1 adipocytes. After nocodazole treatment to disrupt microtubules, GLUT4 vesicles were dispersed from the perinuclear region in the basal state, and insulin-induced GLUT4 translocation was partially inhibited by 20–30%, consistent with other reports. We found that platelet-derived growth factor (PDGF), which did not stimulate GLUT4 translocation in intact cells, was surprisingly able to enhance GLUT4 translocation to ~50% of the maximal insulin response, in nocodazole-treated cells with disrupted microtubules. This effect of PDGF was blocked by pretreatment with wortmannin and attenuated in cells pretreated with cytochalasin D. Using confocal microscopy, we found an increased co-localization of GLUT4 and F-actin in nocodazole-treated cells upon PDGF stimulation compared with control cells. Furthermore, immunoejection of small interfering RNA targeting the actin-based motor Myo1c, but not the microtubule-based motor Kif3A, significantly inhibited both insulin- and PDGF-stimulated GLUT4 translocation after nocodazole treatment. In summary, our data suggest that 1) proper perinuclear localization of GLUT4 vesicles is a requirement for insulin-specific stimulation of GLUT4 translocation, and 2) nocodazole treatment disperses GLUT4 vesicles from the perinuclear region allowing them to engage insulin and PDGF-sensitive actin filaments, which can participate in GLUT4 translocation in a phosphatidylinositol 3-kinase-dependent manner.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal anti-GLUT4 antibody was purchased from Chemicon International (Temecula, CA). Monoclonal anti-KAP3A antibody was from Transduction Laboratories (Lexington, KY), and monoclonal anti-β-tubulin antibody was from Sigma. Sheep IgG and rhodamine- , FITC-conjugated anti-rabbit, -mouse, and, -sheep IgG antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). KAP3A, Myo1c, and scrambled siRNAs were purchased from Dharmacon Research, Inc. (Lafayette, CO). Dulbecco’s modified Eagle’s medium and fetal calf serum were purchased from Life Technologies. 2-[3H]Deoxyglucose and 1-[3H]glucose were from ICN (Costa Mesa, CA). Wortmannin, LY294002, jasplakinolide, and PD98059 were from Calbiochem. Oregon-green phalloidin was from Molecular Probes (Eugene, OR). Nocodazole, colchicine, cytochalasin D, insulin, PDGF, and other reagents were purchased from Sigma.

Cell Culture and Treatment—3T3-L1 cells were cultured and differentiated as described previously (16). Differentiated cells were serum-starved for 2–3 h, pretreated at the same time with or without 33 μM nocodazole for 2 h, 10 μM colchicine for 3 h, 2 μM cytochalasin D for 3 h, 10 μM jasplakinolide for 2 h, or vehicle MeSO for 2–3 h, followed by stimulation with or without insulin or PDGF.

Microinjection—Microinjection was performed using a semiautomatic Eppendorf Microinjection system. The siRNAs were designed to

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4 The abbreviations used are: PDGF, platelet-derived growth factor; siRNA, small interfering RNA; GLUT4, glucose transporter isof orm 4; 2-DG, 2-deoxyglucose; FITC, fluorescein isothiocyanate; PI3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B.
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FIGURE 1. Effects of PDGF on GLUT4 translocation and 2-Dog uptake in 3T3-L1 adipocytes treated with nocodazole. A, after the pretreatment with or without 33 μM nocodazole (Noc) for 2 h, 10 μM colchicine (Colch) for 3 h, 2 μM cytochalasin D (CD) for 3 h, 10 nM jasplakinolide (JPN) for 2 h, or 0.1% Me2SO vehicle for 2-3 h, serum-starved cells were stimulated with or without 100 μg/ml insulin or 50 ng/ml PDGF for 20 min. Cells were then fixed and processed for immunostaining as described below. B, after the pretreatment with or without 33 μM nocodazole for 2 h, 10 μM colchicine for 3 h, 2 μM cytochalasin D for 3 h, 10 μM jasplakinolide for 2 h, or 0.1% Me2SO vehicle for 2-3 h, serum-starved cells were stimulated with or without 100 ng/ml insulin or 50 ng/ml PDGF for 20 min, followed by the measurement of 2-Dog uptake. The data represent the means ± S.E. from three independent experiments.
tion by microtubule disruption raises the possibility that actin filament-based transport can mediate GLUT4 vesicle movement to the cell surface following the dispersal of GLUT4 vesicles.

To test the above hypothesis, we treated cells with 50 ng/ml (2 nM) PDGF after pretreatment with nocodazole or colchicine with or without cytochalasin D or jasplakinolide. As we (14) and others (15) have reported previously, PDGF did not stimulate GLUT4 translocation in intact cells. Interestingly, after nocodazole or colchicine treatment, we found that PDGF stimulation increased the cell surface GLUT4 level to ~50% of the maximal insulin effect ($p < 0.05$) and that this effect of PDGF was markedly attenuated in cells pretreated with both nocodazole/colchicine and cytochalasin D/jasplakinolide (Fig. 1A). These results support the hypothesis that after GLUT4 vesicles are dispersed from their normal perinuclear localization into the cytoplasm, PDGF is now able to stimulate GLUT4 translocation as long as the actin cytoskeleton response is intact, consistent with an F-actin mediated mechanism.

We also conducted comparable 2-DOG uptake experiments to study the effect of PDGF on glucose transport. As seen in Fig. 1B, insulin caused a 7.8-fold increase in 2-DOG uptake, whereas PDGF had only a very slight effect, consistent with previous reports that PDGF does not stimulate glucose transport (14, 15). In contrast, when cells were pre-

**FIGURE 2.** The stimulatory effect of PDGF on GLUT4 translocation in nocodazole-treated cells is PI3-kinase-dependent. After the pretreatment with or without 33 μM nocodazole (Noc) for 2 h, together with or without incubation with 100 nM wortmannin (WM) for 30 min, 50 μM LY294002 (LY) for 30 min, 50 μM PD98059 (PD) for 30 min, or 0.1% Me2SO (DMSO) vehicle for 2–3 h, serum-starved cells were stimulated with or without 10 ng/ml insulin or 50 ng/ml PDGF for 20 min. Cells were then fixed and immunostained for GLUT4, and GLUT4 translocation was evaluated using ring assay by fluorescence microscopy (A). The data represent the means ± S.E. from three independent experiments. Plasma membrane sheets were prepared and GLUT4 (B) or GLUT1 (D) was visualized by immunofluorescence microscopy as described under “Experimental Procedures.” The representative images were shown from three independent experiments. The intensities of GLUT4 (C) or GLUT1 (E) immunofluorescence on the plasma membrane sheets (B, D) were quantitated using Simple-PCI software. The results were obtained from 35–50 cells in each condition, and the data represent the means ± S.E.
We pretreated serum-starved cells with nocodazole for 2 h, serum-starved cells were stimulated with or without 10 ng/ml insulin or 50 ng/ml PDGF for 15 min. Cells were then fixed and immunostained with anti-GLUT4 antibody, followed by incubation with rhodamine-conjugated secondary antibody. F-actin was stained with Oregon-green phalloidin. Localization of GLUT4 and F-actin was examined using confocal microscopy.

The stimulatory effect of PDGF on GLUT4 translocation is PI3-kinase-dependent—We pretreated serum-starved cells with 33 μM nocodazole or vehicle for 2 h, followed by treatment with PI3-kinase inhibitors (wortmannin (100 nM) or LY294002 (50 μM)) for 30 min, or the MEK inhibitor PD98059 (50 μM) for 30 min, prior to insulin or PDGF stimulation. As shown in Fig. 2A, after nocodazole treatment, insulin and PDGF caused a 165 and 67% increase in GLUT4 translocation, respectively. Although there is no statistical significance in insulin-induced GLUT4 translocation between Me2SO and nocodazole pretreatment, PDGF significantly increased GLUT4 translocation in nocodazole-treated cells (p < 0.05). The stimulatory effects of insulin and PDGF were completely inhibited by wortmannin or LY294002 treatment, but PD98059 treatment had no effect. These results show that PDGF-stimulated and insulin-stimulated GLUT4 translocation, after nocodazole treatment, is PI3-kinase-dependent but not MEK-dependent.

To confirm these results with another method, we performed similar experiments using the plasma membrane sheet assay to measure GLUT4 translocation. As shown in Fig. 2B, there was a significant increase in GLUT4 immunofluorescence on the plasma membrane sheets upon PDGF stimulation in cells treated with nocodazole compared with control vehicle-treated cells. The PDGF stimulatory effect was completely inhibited by pretreatment with wortmannin (100 nM, 30 min), but pretreatment with PD98059 had no effect. As shown in Fig. 2C, the intensities of GLUT4 immunofluorescence on the plasma membrane sheets (as quantitated using SimplePCI software (C-Imaging Systems)) are comparable with the GLUT4 translocation data measured by immunofluorescence microscopy in Fig. 2A.

On the other hand, plasma membrane GLUT1 expression, as assessed by the plasma membrane sheet assay, was increased 1.8-fold by insulin stimulation but was not significantly affected by microtubule disruption (Fig. 2, D and E), consistent with previous reports (4, 6). PDGF did not significantly affect PM GLUT1 expression either with or without nocodazole treatment.

Actin-based Transport Can Translocate GLUT4 Vesicles after Disruption of Microtubules—We postulated that after nocodazole-induced microtubule disruption, dispersed GLUT4 vesicles can engage actin filaments allowing PDGF to stimulate GLUT4 translocation through an F-actin-mediated mechanism (Fig. 1). To explore this possibility, we examined the co-localization of GLUT4 and actin filaments using confocal microscopy. As shown in Fig. 3, in basal cells pretreated with Me2SO, GLUT4 vesicles were localized predominantly to the perinuclear region, and the F-actin structure was poorly discriminated (time 0). After insulin stimulation, GLUT4 vesicles are translocated to the plasma membrane, displaying a strong fluorescence ring, cortical actin filaments were now clearly formed, and there was co-localization of GLUT4 and F-actin (overlay). This observation is consistent with studies showing co-localization of translocated GLUT4 with rearranged actin after insulin treatment in L6 myotubes (22, 23). In contrast, after PDGF stimulation, the majority of the GLUT4 vesicles remained in a perinuclear location, but there was marked membrane ruffling indicative of PDGF-induced cortical actin rearrangement. There was little co-localization of GLUT4 and F-actin (Fig. 3).

In basal cells pretreated with nocodazole, GLUT4 vesicles were dispersed from the perinuclear region, and were now localized throughout the cytoplasm. Upon insulin stimulation, cortical actin filaments became clearly visible, a circumferential ring of GLUT4 could be detected, which co-localized with F-actin in the cortical region of the cell (Fig. 3). Similarly, the PDGF stimulation of nocodazole-treated cells resulted in GLUT4 translocation to the cell surface, formation of visible cortical actin structures, and co-localization of GLUT4 with F-actin. These results suggest that after disruption of the microtubule network with nocodazole, the dispersed GLUT4 vesicles can be recruited to the plasma membrane via actin filaments with PDGF or insulin stimulation.

Motor proteins participate in vesicle trafficking by moving cargo along the cytoskeleton (1). We have recently shown that the motor protein kinesin KIF3 plays an important role in insulin-induced GLUT4 transport.
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exocytosis in 3T3-L1 adipocytes (9), and another kinesin family member, KIF5, has also been implicated in this process (10). Besides microtubule-associated motor proteins, the actin motor protein, Myo1c, has recently been shown to facilitate GLUT4 transport in response to insulin in 3T3-L1 adipocytes (24). If the actin system can mediate GLUT4 translocation in the absence of intact microtubules, the actin motor Myo1c may be responsible for transporting GLUT4 vesicles under these conditions. To examine the roles of motor proteins in GLUT4 translocation with or without microtubule disruption, we conducted single cell microinjection experiments. As seen in Fig. 4A, microinjection of Myo1c-siRNA into cells pretreated with Me2SO significantly inhibited insulin-induced GLUT4 translocation by 65%, consistent with a previous study showing a role for Myo1c in insulin-induced GLUT4 translocation (24). Microinjection of Myo1c-siRNA into nocodazole-pretreated cells significantly inhibited not only insulin- but also PDGF-stimulated GLUT4 translocation by ~50 and ~85%, respectively. This same Myo1c-siRNA has been shown to knock down protein expression, as well as inhibit 2-DOG uptake when electroporated into 3T3-L1 adipocytes (24).

We also examined the role of KIF3 in this system by microinjecting an antibody against KAP3A, a binding subunit of KIF3. Consistent with our previous report (9), KAP3A antibody injection inhibited insulin-induced GLUT4 translocation in control cells (Fig. 4B, lanes 2 and 4 from left side; p < 0.05). However, it did not significantly affect either insulin- or PDGF-stimulated GLUT4 translocation in nocodazole-treated cells in which the microtubule system had been disrupted (Fig. 4B, lanes 5–10 from left side). Similar results were obtained when KAP3A-siRNA was injected (Fig. 4C). These results further support the idea that the actin network can mediate GLUT4 translocation after GLUT4 vesicles are dispersed throughout the cytoplasm from their normal perinuclear location by microtubule disruption.

Insulin- and PDGF-induced Akt/PKB Activation Is Not Affected by Disruption of Microtubules—To determine whether the initial signaling events of PDGF-stimulated GLUT4 translocation were affected by microtubule disruption, we measured the insulin and PDGF activation of Akt/PKB. As seen in Fig. 5, both insulin (100 ng/ml, 10 min) and PDGF (50 ng/ml, 10 min) led to phosphorylation of Akt Ser-473 with or without nocodazole or colchicine, although the PDGF effects are only 20% of the maximal insulin response, consistent with our (14) and other (25) previous reports. These results show that microtubule disruption does not affect these aspects of insulin and PDGF signaling and clearly does not result in enhanced PDGF-mediated Akt activation.

DISCUSSION

Recent studies reveal a role for the intact microtubule network in the process of GLUT4 translocation (26); however, it remains somewhat controversial. For example, several studies have suggested that an intact microtubule network is necessary for insulin-stimulated GLUT4 translocation. Thus, disruption of microtubules led to inhibition of insulin-stimulated glucose uptake and GLUT4 translocation (4, 7, 8, 27). Inhibition of the microtubule-based motor proteins dynein or kinesin decreased insulin-stimulated GLUT4 translocation in adipocytes (9, 18, 28). In contrast, other groups have reported that insulin-stimulated GLUT4 translocation is microtubule-independent (11–13) and that nocodazole inhibits insulin-stimulated glucose transport via a microtubule-independent mechanism (12, 13). The differences between these reports have not been well resolved. The current study directly addresses this issue, and we have hypothesized that after microtubules are disrupted by nocodazole or colchicine, GLUT4 vesicles become dispersed and can now reside in the vicinity of the cortical actin network, which participates in the process of GLUT4 translocation to the plasma membrane in response to insulin. Using confocal microscopy, we show co-localization of actin filaments with GLUT4 vesicles in 3T3-L1 adipocytes upon insulin stimulation after nocodazole treatment (Fig. 3). In addition, microinjection of siRNA targeted to the actin-associated motor protein Myo1c, but not the microtubule-associated motor KIF3, significantly inhibited insulin-stimulated GLUT4 translocation in cells pretreated with nocodazole (Fig. 4). These data indicate that after disruption of microtubules, insulin-stimulated translocation of the dispersed GLUT4 vesicles can occur through the actin network and that this is now independent of microtubule function.

Membrane ruffling due to cortical actin rearrangement is PI3-kinase-dependent and plays an important role in insulin-induced GLUT4...
translocation (29, 30). Like insulin, PDGF can also stimulate activation of PI3-kinase with subsequent membrane ruffling (29, 31), but PDGF has no significant stimulatory effect on GLUT4 translocation and glucose transport in intact 3T3-L1 adipocytes (14, 32). Based on our results with insulin stimulation in nocodazole-treated cells, it seemed possible that PDGF, which can induce cortical actin rearrangement, might also be able to stimulate translocation of dispersed GLUT4 vesicles after microtubule disruption. To test this idea, we stimulated nocodazole-treated cells with PDGF and measured 2-DOG uptake and GLUT4 translocation (Fig. 1). The results showed that PDGF was now able to stimulate glucose transport and GLUT4 translocation in these cells, and the stimulatory effect of PDGF was PI3-kinase-dependent. These findings further support the idea that the actin network can mediate the translocation of dispersed GLUT4 vesicles to the cell surface. The PI3-kinase dependence of the PDGF effect after nocodazole treatment is consistent with reports showing that PDGF-induced membrane ruffling is PI3-kinase-dependent (30, 31) and that PDGF stimulates glucose transport in a PI3-kinase-dependent manner in 3T3-L1 adipocytes that overexpress PDGF receptors (33).

Regarding early signaling events following microtubule disruption, we found that neither insulin- nor PDGF-induced Akt/PKB phosphorylation was affected by pretreatment with nocodazole or colchicine (Fig. 5). The magnitude of the PDGF effect on Akt phosphorylation was ~20% of the maximal insulin response, consistent with the previous reports (14, 25). However, there is some controversy on this point; for example, Hill et al. (34) reported that PDGF does not stimulate Akt2/PKB activation, whereas others found that PDGF-induced Akt2/PKB activation as assessed by a kinase activity assay (32). Because it has been shown that there is heterogeneity of PDGF receptor expression with 3T3-L1 adipogenesis (15), this could possibly explain these differences.

Taken together, it is suggested that in the presence of an intact cytoskeletal network, both microtubules and actin filaments participate in insulin-induced GLUT4 translocation. Perinuclear GLUT4 vesicles, anchored by the microtubule structure, are first transported by microtubule-associated motor proteins, such as kinesins, along the microtubules to the cell periphery, where the actin-associated motor myosins can then transport the GLUT4 vesicles along the cortical actin filaments to the plasma membrane. When microtubules are disrupted, the perinuclear GLUT4 vesicles are dispersed throughout the cytoplasm allowing them to gain access to the F-actin system. The dispersed GLUT4 vesicles can be transported directly by myosins along the actin filaments to the plasma membrane. In this case, PDGF, which normally does not stimulate GLUT4 translocation, can enhance GLUT4 vesicle movement to the cell surface by stimulating actin rearrangement. In the same vein, insulin can also induce GLUT4 translocation after microtubule disruption.

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Insulin is unique among hormones and growth factors in its ability to stimulate glucose transport and GLUT4 translocation in muscle and adipose tissues, but the mechanisms for this insulin specificity are still unclear. Although many growth factors can stimulate PI3-kinase activity, it has been suggested that the lack of, or the low number of, receptors for other growth factors, such as PDGF, may account for their inability to activate glucose transport (15, 33). Alternatively it has also been proposed that insulin and PDGF have different compartmental effects on the activation of PI3-kinase (35) and the generation of PIP3 (36), which may also be a mechanism for insulin specificity. Our study points out that the localization of GLUT4 vesicles is also an important factor. We propose that the normal perinuclear localization of GLUT4 vesicles is one of the factors contributing to insulin-specific GLUT4 translocation, because insulin is necessary to overcome the mechanisms that lead to retention of GLUT4 vesicles in this compartment. It is possible that insulin acts at the microtubule level to begin the process of GLUT4 translocation but also has effects on the actin skeletal network, which is needed to continue this process. This would explain why insulin still causes translocation in the presence of nocodazole. If PDGF exerts its effects only at the level of the actin network, this would explain why PDGF can cause GLUT4 translocation in microtubule-disrupted cells with dispersed GLUT4 vesicles but has only weak effects in control cells. In summary, these data suggest that the anchoring of GLUT4 vesicles in the perinuclear storage pool is important for insulin-specific effects on GLUT4 translocation and that both microtubule- and actin filament-based transport systems participate in insulin-induced GLUT4 translocation.

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