Neomycin Prevents the Wortmannin Inhibition of Insulin-stimulated Glut4 Translocation and Glucose Transport in 3T3-L1 Adipocytes*

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Insulin stimulates the movement of the facilitative glucose transporter protein Glut4 (Glut4) from an intracellular compartment to the plasma membrane in adipocytes and muscle cells, resulting in an increased rate of glucose uptake. Insulin-stimulated Glut4 translocation and glucose transport are abolished by wortmannin, a specific inhibitor of phosphatidylinositol 3'-kinase (PI3K). Here, we demonstrate that neomycin, a drug that masks the cellular substrate of PI3K, phosphatidylinositol 4,5-bisphosphate (PIP2), prevents wortmannin inhibition of insulin-stimulated Glut4 translocation and glucose transport without activating protein kinase B, a downstream effector of PI3K. These results suggest that PIP2 may have an important regulatory function in insulin-stimulated Glut4 translocation and glucose transport.

Skeletal muscle and adipose tissue are the major sites of insulin-stimulated glucose disposal. Reduced insulin-stimulated glucose uptake by skeletal muscle or adipose tissue is of major importance in several common human disorders including type 2 diabetes, obesity, hypertension, and combined hyperlipidemia (1).

Insulin promotes glucose transport into fat and muscle cells by stimulating the translocation and fusion of intracellular Glut4 storage vesicles with the plasma membrane (2). Insulin-stimulated Glut4 translocation to the cell surface requires the co-ordinated activity of numerous intracellular target molecules (3). One key intracellular target activated by insulin is phosphatidylinositol 3-kinase (PI3K), which catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) (4, 5). Insulin stimulation of PI3K activity is essential for insulin-stimulated glucose transport (4–6), and the specific PI3K inhibitor, wortmannin, abrogates PIP3 production, Glut4 translocation, and glucose transport (6–8). Recent evidence now suggests that while PI3K activity is necessary, other insulin-dependent pathways are also required to achieve glucose transport (9–11). Recent work by the groups of Saltiel and Pessin (11, 12) have identified several components of a PI3K-independent signaling pathway required for insulin-stimulated glucose transport; these components include CAP, c-Cbl, and the small GTPase, TC10.

The large majority of current research into the insulin-stimulated PI3K signaling pathway focuses almost exclusively on molecules activated as a result of PI3K-mediated PIP3 production (13–15). These studies have been key in the discovery of molecules like protein kinase B (PKB) that link insulin signaling to its effects on glycogen and protein synthesis and gene transcription (14, 16, 17). However, PKB involvement in glucose transport is still hotly debated, with conflicting evidence for and against such an involvement (18–20). In contrast to the intense interest in the role of PIP3 in insulin action, there has been much less characterization of PIP2 function in insulin-stimulated glucose transport. The integral involvement of PIP2 in vesicle trafficking and actin remodelling events are well documented (reviewed in Ref. 21), and these two events are essential if Glut4 vesicles are to move from an intracellular compartment to the plasma membrane. Insulin stimulation of PI3K activity will lead to a loss of PIP2, as a result of its conversion to PIP3 (22). In addition, insulin has been shown to stimulate phospholipase C (PLC) activity in 3T3-L1 adipocytes; PLC catalyzes the breakdown of PIP2 generating diacylglycerol and inositol 1,4,5-trisphosphate (IP3) (23). Thus, insulin stimulation of adipocytes will promote a loss of PIP2 as a result of conversion to PIP3 and breakdown to DAG and IP3. Here, we have analyzed the function of the substrate of PI3K activity, PIP2, in insulin-stimulated glucose transport. To assess the role of PIP2 in insulin action, we have examined the effects of neomycin, an aminoglycoside that binds and masks PIP2 (24, 25), on insulin-stimulated Glut4 translocation and glucose transport. Remarkably, neomycin was found to prevent wortmannin inhibition of insulin-stimulated glucose transport and Glut4 translocation without effect on PKB phosphorylation or activity. These results suggest that an important requirement for efficient insulin-stimulated glucose transport is the loss or “masking” of PIP2.

MATERIALS AND METHODS

Reagents—Fetal bovine serum was purchased from Sigma (Poole, Dorset, UK). Dulbecco’s modified Eagle’s medium (DMEM) was from Invitrogen (Paisley, UK). Antibodies against PKB and Ser1172-phosphorylated PKB were from Cell Signaling, New England Biolabs (Hertfordshire, UK). PKB substrate peptide (KRPRATATPF) was a kind gift from Dr. R. Plevin (University of Strathclyde, Glasgow, UK). 2-Deoxy-D-3H-glucose was purchased from PerkinElmer Life Sciences (Hertfordshire, UK). Wortmannin and neomycin were purchased from Sigma. Microcystin-LR was a kind gift from Dr. Greg Murphy (GlaxoSmithKline, Harlow, UK). Porcine insulin was a kind gift from NovoNordisk.

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Cell culture—3T3-L1 fibroblasts were grown in DMEM containing 10% calf serum at 37 °C in a humidified atmosphere of 10% CO₂. For differentiation into adipocytes, fibroblasts were grown to confluence, and the medium was replaced with DMEM containing 10% fetal calf serum, 250 mM dexamethasone, 110 μg/ml isobutylmethylxanthine, and 172 mM insulin for 2 days. Medium was then replaced with DMEM containing 10% fetal calf serum and 172 mM insulin for a further 2 days. Adipocytes were used between 10 and 12 days following differentiation.

2-Deoxy-D-glucose Transport Assays in Cultured 3T3-L1 Adipocytes—Differentiated adipocytes were incubated in serum-free medium for 2 h in the presence or absence of 10 μM neomycin. For neomycin-treated cells, neomycin was maintained at a concentration of 10 μM throughout the experiment. The serum-starved cells were washed twice in 2 ml/well of 2:1 (v/v) Hanks-Ringer-Hepes buffer (25 mM Hepes, pH 7.4, 118 mM NaCl, 5 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 2.5 mM CaCl₂). The cells were then incubated for 30 min at 37 °C in the presence or absence 1 μM insulin; wortmannin-treated cells were incubated with 100 nM wortmannin for a total of 40 min, including 10 min prior to the addition of insulin. [³H]-2-Deoxyglucose (at a final concentration of 10 μCi/ml) was added to each well for 4 min, and the cells were quickly washed in ice-cold PBS and solubilized in Triton X-100, and [³H]-2-deoxy-D-glucose uptake was measured by scintillation counting. Glut-specific glucose uptake was measured by subtracting values for [³H]-2-deoxy-D-glucose uptake in the presence of 10 μM cytochalasin B.

Immunoblot Analysis—Cells were solubilized in lysis buffer (50 mM Hepes, pH 7.4, 5 mM NaF, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 50 mM mannitol, 1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 2 μM microcystin-LR, 1% Triton X-100). Lysates were centrifuged for 10 min at 14,000 rpm. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using specific antibodies. Antibody binding was detected using the enhanced chemiluminescence (Amersham Biosciences, Bucks, UK) according to manufacturer's instructions.

Immunofluorescence Analysis of Plasma Membrane Lawns—3T3-L1 adipocytes were cultured on collagen-coated glass coverslips. Cells were serum-starved in the presence or absence of 10 μM neomycin for 2 h, followed by stimulation with 1 μM insulin (in the presence or absence of 100 μM wortmannin as indicated in the figure legends; wortmannin was added to the well 10 min prior to insulin treatment) for 20 min. Plasma membrane lawns were prepared as described in Ref. 26. Cells were transferred onto ice and washed three times with ice-cold PBS and fixed in 4% paraformaldehyde. Cells were washed three times with PBS, followed by three washes in PBS, 5% goat serum over a time period of 15 min. Following this, cells were incubated with primary antibody diluted in PBS, 1% goat serum for 1 h. After this time, cells were washed three times with PBS, 1% goat serum and incubated with fluorescein isothiocyanate-conjugated secondary antibodies diluted in PBS, 1% goat serum for 1 h. Finally, cells were washed with five changes of PBS over a 15-min period and mounted on glass slides and visualized using a Zeiss Pascal on an Axiovert microscope using X40.

RESULTS

Neomycin Restores the Insulin-stimulated Glucose Transport Inhibited by the PI3K Inhibitor Wortmannin—To analyze the possible role of PIP₂ in insulin-stimulated glucose transport, we examined the effect of the aminoglycoside, neomycin, on glucose transport into 3T3-L1 adipocytes. This agent was chosen as it binds with high specificity to PIP₂ (24, 25) and has been used previously in several studies examining PIP₂ function (28–30). The results in Fig. 1A show that 10 μM neomycin had no significant effect on the basal level of glucose transport compared with vehicle. Incubation of adipocytes in 100 nM insulin gave rise to a characteristic 10-fold increase in glucose transport (Fig. 1A). Neomycin treatment caused a small (24 ± 10%) but significant inhibition of insulin-stimulated glucose transport (p = 0.05). As expected, insulin-stimulated glucose transport was completely abolished by the PI3K inhibitor, wortmannin (Fig. 1A). However, remarkably, this inhibition of glucose transport by wortmannin was restored by 47 ± 13%, p < 0.005 in the presence of neomycin (Fig. 1A). Neomycin prevented wortmannin inhibition of insulin-stimulated glucose transport in a dose-dependent manner (Fig. 1B). These results suggest that an important aspect of insulin-stimulated PI3K activation is the subsequent loss or masking of PIP₂.

To rule out any interfering effects of neomycin on insulin signaling or on the ability of wortmannin to inhibit PI3K, the phosphorylation and activity of the PI3K effector, PKB, was assessed. As shown in Fig. 2A and quantified in Fig. 2B, in both the presence and absence of neomycin, insulin stimulation results in the significant phosphorylation of serine (S) at position 473 in PKB. Thus, such phosphorylation data does not consider effects on PKB activity.
under the same treatment conditions used previously, was measured. As shown in Fig. 2C, insulin stimulated a marked rise in PKB activity; the magnitude of this response was similar in neomycin-treated cells (Fig. 2C). As expected, wortmannin treatment caused a marked inhibition of insulin-stimulated PKB activity (54 ± 3%). In agreement with the results of the PKB phosphorylation data (Fig. 2, A and B), neomycin did not prevent the wortmannin-induced inhibition of insulin-stimulated PKB activity (Fig. 2C). Thus, the reversal of wortmannin-inhibited insulin-stimulated glucose transport is independent of any effects of neomycin on PKB activation.

Neomycin Prevents Wortmannin Inhibition of Insulin-dependent Glut4 Translocation to the Plasma Membrane—The results presented in Fig. 1, A and B, clearly demonstrate that neomycin prevents the inhibition of insulin-stimulated glucose transport elicited by wortmannin. As the increased rate of glucose transport promoted by insulin is largely a result of translocation of Glut4 from an intracellular storage compartment to the plasma membrane, we examined the effect of neomycin treatment on Glut4 insertion into the plasma membrane. For this, plasma membrane sheets derived from 3T3-L1 adipocytes, prepared by sonication of cells attached to cover slips, were stained with Glut4 antibody as described under “Materials and Methods.” Fig. 3A shows that plasma membrane sheets prepared from unstimulated cells have only very low levels of Glut4 staining in both the presence and absence of neomycin. Insulin elicited a marked increase in plasma membrane Glut4 content, and interestingly this was further enhanced by neomycin (53 ± 4% increase, p = 0.05, n = 3). As expected, wortmannin treatment resulted in a marked decrease in the insulin-dependent increase in Glut4 levels at the plasma membrane. However, consistent with the glucose transport data, the presence of neomycin prevented wortmannin inhibition of the insulin-dependent increase in plasma membrane Glut4 content. Quantification of the fluorescence intensity from 30 fields of view per condition is shown in Fig. 3B.

**DISCUSSION**

It is widely accepted that the lipid kinase PI3K plays an essential role in insulin-stimulated glucose transport (4, 6, 22, 31). This is borne out by the complete inhibition of insulin-stimulated glucose transport by the fungal metabolite and specific inhibitor of PI3K, wortmannin (6). Intensive research into the downstream effectors of PI3K activity involved in insulin signaling have resulted in the identification of molecules such as protein kinase B and atypical PKCs that bind PIP3 (13, 32). However, the exact requirement for these kinases in insulin-stimulated glucose transport is unresolved. Indeed, although PI3K activity is necessary for insulin-stimulated Glut4 trans-
location, the role of PIP₂ in this process is not clear. For example, overexpression of the pleckstrin homology domains of GRP-1 or PKB that bind specifically to PIP₂ do not prevent insulin-dependent Glut4 translocation and insertion into the plasma membrane (31, 33). Furthermore, expression of a dominant-negative mutant of the lipid phosphatase PTEN that increased PIP₂ levels had no effect on either basal or insulin-stimulated glucose transport (34).

In this study, we find that treatment of cells with the angiotensin II receptor antagonist losartan preserves the insulin-dependent inhibition of unstimulated glucose transport. Neomycin has this effect without influencing the phosphorylation state or activity of PKB, a downstream effector of PI3K, demonstrating that the effects of neomycin are not due to interference with wortmannin action. In addition to the effects on glucose transport, neomycin also prevented wortmannin inhibition of insulin-stimulated Glut4 translocation. Interestingly, assessment of Glut4 translocation reveals that the insulin-dependent plasma membrane Glut4 content is increased in the presence of neomycin compared with insulin alone. This increase in insulin-dependent Glut4 content in the presence of neomycin is in contrast with the modest inhibition of insulin-stimulated glucose transport under the same conditions. This difference is also observed between the insulin-stimulated glucose transport rates and Glut4 translocation data from wortmannin-treated cells in the presence of neomycin. Such a difference may indicate that masking of PIP₂ by neomycin as well as stimulating Glut4 translocation also has a downstream effect on the activity of the glucose transporter. Nevertheless, the data are consistent in that neomycin prevents the wortmannin-induced inhibition of both insulin-stimulated glucose transport and Glut4 translocation.

It is interesting to speculate as to the mechanism by which neomycin elicits these effects on Glut4 translocation and glucose transport. Neomycin is characterized by an affinity to bind phosphoryositides in particular, PIP₂ (24, 25). Binding of neomycin to PIP₂ results in the masking of this lipid (25, 29). Taken together with our observations, it may be reasoned that the dynamic turnover of PIP₂ that takes place on activation of PI3K may be an important signal in stimulating glucose transport. It is important to spell out what dynamic turnover means; in this context we use it to describe a rapid change in the real time depletion and resynthesis of PIP₂ brought on by agonist stimulation. The idea that stimulus-dependent dynamic turnover of PIP₂ can affect exocytosis has recently been described for insulin secretion by MIN6 cells (35) and human growth hormone release in PC12 cells (36). In both these systems the involvement of ADP-riboseylation factor-6 in the maintenance of PIP₂ levels was important in exocytosis. More directly, other agonists like endothelin-1 and thrombin can elicit an increase in glucose transporter translocation to the plasma membrane and glucose transport, although not to the same extent as insulin (37–39). Both endothelin-1 and thrombin receptor agonist result in dramatic decreases in PIP₂ (40, 41); in the case of endothelin-1 this is believed to involve PLC activation and subsequent cleavage of PIP₂ to yield IP₃ and diacylglycerol (40). The ability of neomycin to bind PIP₂ may mimic the depletion of PIP₂ which together with other insulin-dependent signals may be sufficient to stimulate Glut4 translocation and glucose transport. Finally, this study suggests that insulin-stimulated Glut4 translocation can proceed in the absence of PKB activation.

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