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# PI 3-KINASE ACTIVATION IS REQUIRED FOR INSULIN STIMULATION OF GLUCOSE TRANSPORT INTO L6 MYOTUBES

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Phosphatidylinositol 3-kinase (PI 3-kinase) is acutely stimulated by insulin but its role in regulating glucose metabolism is still not fully understood. Insulin acutely stimulates glucose transport into L6 myotubes approximately 2-fold. and activates PI 3-kinase activity 2 to 3-fold. Wortmannin, an inhibitor of PI 3-kinase, blocked insulin stimulation of 2-deoxyglucose transport into the myotubes in a time and dose-dependent manner. Inhibition was observed within 5 minutes and was complete by 30 minutes.The $IC_{50}$ for this inhibition was ~10 nM; almost complete inhibition was observed at 100 nM. Similarly, insulin stimulation of PI 3-kinase activity was inhibited by wortmannin in a dose-dependent manner. The insulinmimetic vanadate activated hexose transport into the myotubes to more than 50% of the maximal level attained with insulin. Only ~60% of vanadate-activated glucose transport was inhibited by maximal wortmannin concentrations. It is concluded that insulin activation of PI 3-kinase is necessary for stimulation of glucose transport into L6 muscle cells. In contrast, vanadate appears to augment transport by acting upon PI 3-kinase-dependent and independent pathways. 

Skeletal muscle is the major site of post-prandial glucose uptake from the blood (1). Insulin activates glucose transport into skeletal muscle *in vivo* and *in vitro* (2). When differentiated into myotubes, L6 muscle cells possess many of the morphological and biochemical properties of mature skeletal muscle tissue (3). Included in this repetoire, is the ability of insulin to acutely stimulate glucose transport into the cells (4, 5). Such activation occurs primarily by the translocation of glucose transporters from intracellular vesicles to the plasma membrane thereby increasing the Vmax of the transport process (6). The insulin signaling pathway by which this process is regulated is not fully understood.

Upon binding to its receptor, insulin activates the insulin receptor tyrosine kinase (IRTK) (7). This results in the phosphorylation of the receptor's immediate

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downstream effector, insulin receptor substrate-1 (IRS-1) (8). The tyrosine-phosphorylated IRS-1 then binds to the SH2 domains of the p85 regulatory subunit of PI 3-kinase causing activation of the enzyme's p110 catalytic subunit (9). PI 3-kinase phosphorylates phosphatidylinositol, phosphatidylinositol-4-monophosphate and phosphatidylinositol-4,5-biphosphate at the D-3 position to produce phosphatidylinositol-3-monophosphate, phosphatidylinositol-3,4-biphosphate and phosphatidylinositol-3,4,5-triphosphate, respectively (10, 11). The precise physiological roles of the D-3 phosphorylated phosphoinositides have not been determined, although, it has been shown that they play a role in regulating cytoskeleton structure by augmenting polymerization of actin (12). In addition, a homologue of the PI 3-kinase catalytic subunit found in yeast appears to be required for proper protein sorting (13). Most recently, it has been demonstrated that activation of PI 3-kinase is necessary for insulin stimulation of glucose transport into primary rat adipocytes and 3T3-L1 adipocytes (14, 15).

Here, we have examined the role of PI 3-kinase in the regulation of glucose transport into L6 myotubes. It was found that insulin acutely stimulates 2-deoxyglucose transport and PI 3-kinase activity in these muscle cells. Wortmannin, an inhibitor of PI 3-kinase, blocked insulin stimulation of glucose transport in a time and dose-dependent manner. In parallel, the compound also inhibited insulin-augmented PI 3-kinase activity in a dose-dependent manner. Vanadate stimulated hexose transport into the myotubes to ~50% the maximal level attained with insulin. Wortmannin maximally inhibited vanadate-stimulated transport only ~60%. It is concluded that PI 3-kinase is an element in the insulin signaling pathway that regulates glucose transport into L6 myotubes and that activation of PI 3-kinase is necessary for acute stimulation of transport by the hormone. In contrast, the insulinmimetic vanadate appears to stimulate glucose transport by acting upon elements in PI 3-kinase-dependent and independent pathways.

## MATERIALS AND METHODS

**Materials:** Wortmannin, 2-deoxy-D-glucose, phloretin, cytochalasin B and anti-phosphotyrosine antibodies conjugated to agarose beads were purchased from Sigma. Porcine insulin was from Elanco. Bovine serum albumin (BSA), fraction V, was purchased from ICN Immunobiologicals. Tissue culture reagents were obtained from GIBCO. 2-Deoxy-D-[$^3$H]glucose was purchased from New England Nuclear.

**Cell culture:** The skeletal muscle cell line, L6, was kindly provided by Dr. Amira Klip (Division of Cell Biology, The Hospital for Sick Children, Toronto, Canada). Cells were grown in $\alpha$-MEM containing 2% FBS, 100 units/ml Penicillin G and 100 mg/ml Streptomycin sulfate at 37°C in a humidified atmosphere of 8.5% $CO_2$. Cells were allowed to grow and fuse into myotubes as previously described (4, 16).

Prior to experimentation, cells were incubated in growth media supplemented with 20 mM glucose for 24 hours.Cells were then washed 3 times with Krebs-Ringer Phosphate Hepes (KRPH) buffer (150 mM NaCl, 5 mM KCl, 2.9 mM $Na_2HPO_4$, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM Hepes, pH 7.4) supplemented with 0.1% BSA.

**2-Deoxyglucose transport assay:** Washed myotubes were preincubated for 30 min at 37 °C in KRPH buffer ± wortmannin then incubated for an additional 30 min ±100 nM insulin or 10 mM vanadate at 37 °C. Transport of 10 μM 2-deoxy-D-[$^3$H]glucose (0.2 mCi/nmol) was measured for 15 min at 37 °C in a final volume of 200 μl KRPH/0.1% BSA ± insulin or vanadate alone or in the presence of wortmannin as previously described (17). Non-specific transport was defined as that which occurred in the presence of 40 μM cytochalasin B. To determine the time course of wortmannin's action, Cells were preincubated with 100 nM insulin for 30 min at 37 °C then treated with 100 nM wortmannin for the times indicated in the continuing presence of 100 nM insulin at 37 °C. Transport of 2-deoxy-D-[$^3$H]glucose was then determined as described above.

**PI 3-kinase assay:** Washed myotubes were preincubated for 30 min at 37 °C in KRPH buffer ± wortmannin and subsequently stimulated with 100 nM insulin for 5 min at 37 °C. Lysates were prepared and solubilized proteins were subjected to immunoprecipitation with monoclonal anti-phosphotyrosine antibodies conjugated to agarose beads. The PI 3-kinase activity was determined directly in the immunoprecipitates as described previously (18). The reaction was carried out at 25 °C for 10 min in a mixture (50 μl) containing 0.2 mg/ml of PI (sonicated), 20 mM Hepes, pH 7.1, 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM $MgCl_2$, and [$\gamma$-$^{32}$P]ATP (40 μM and 0.1 μCi/μl) and terminated by addition of 15 μl of 4 N HCl and 130 μl of chloroform:methanol (1:1). Tubes were vortexed for 1 min and 30 μl of the lower layer was spotted on a Silica Gel 60 plate (pre-coated with 1% potassium oxalate and activated at 100 °C for 1h). Plates were developed in $CHCl_3$:$CH_3OH$:$H_2O$:$NH_4OH$ (60:47:11.3:2) (19) and visualized with a PhosphorImager (Molecular Dynamics). Radioactivity associated with the spots corresponding to PIP was quantified by Cerenkov counting.

## RESULTS AND DISCUSSION

Insulin maximally stimulates glucose transport into L6 myotubes approximately 2-fold (4, 5). To investigate the role of PI 3-kinase in this insulin signalling cascade, myotubes were acutely stimulated with insulin after being preincubated alone or with increasing concentrations of wortmannin, a fungal metabolite that is a potent inhibitor of PI 3-kinase. As demonstrated in Figure 1, wortmannin effectively antagonized insulin-activated 2-deoxyglucose transport in a dose-responsive manner with an $IC_{50}$ of less than 30 nM. Almost complete inhibition of insulin-stimulated transport occurred at a concentration of 100 nM. These results parallel those previously reported for the inhibitory effects of wortmannin on insulin-stimulated glucose transport into primary rat adipocytes where complete inhibition was also reported to occur at 100 nM (14). Similarly, utilizing the recently synthesized inhibitor of PI 3-kinase, LY294002, it has been

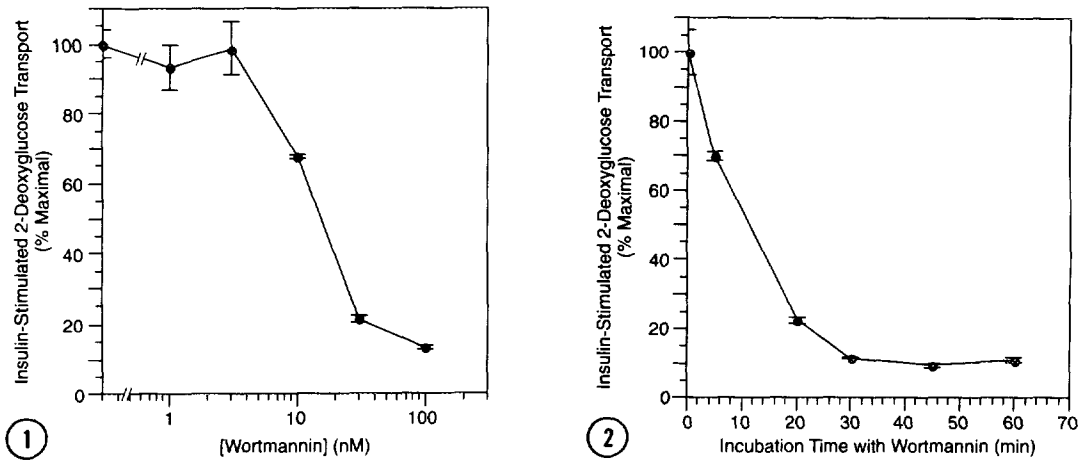


Figure 1. Inhibition by wortmannin of insulin-stimulated 2-deoxy-D-[$^3$H]glucose transport into L6 myotubes. Cells were incubated for 30 min at 37 $^{o}$C with the indicated concentrations of wortmannin. The cells were then treated with 100 nM insulin for an additional 30 min. Transport of 2-deoxy-D-[$^3$H]glucose was measured as indicated in the "Materials and Methods" section. Transport in the presence of 40 μM cytochalasin B has been subtracted. Results presented are the means ± S.E. of triplicate determinations.

Figure 2. Time course of the inhibition by wortmannin of insulin-stimulated 2-deoxy-D-[$^3$H]glucose transport into L6 myotubes. Cells were incubated with 100 nM insulin for 30 min at 37 $^{o}$C then treated with 100 nM wortmannin for the times indicated in the continuing presence of 100 nM insulin at 37 $^{o}$C. Transport of 2-deoxy-D-[$^3$H]glucose was measured as indicated in the "Materials and Methods" section. Transport in the presence of 40 μM cytochalasin B has been subtracted. Results presented are the means ± S.E. of triplicate determinations.

reported that insulin activation of the enzyme is required for stimulation of hexose transport into 3T3-L1 adipocytes (15).

If wortmannin antagonizes insulin-induced glucose transport by inhibiting PI 3-kinase, the onset of the compound's activity should occur in a relatively short period of time. To examine the time course of wortmannin's inhibitory effect, L6 cells were first preincubated with insulin for 30 minutes to obtain maximal stimulation of 2-deoxyglucose transport. The myotubes were then incubated with the compound for varying lengths of time and transport into the cells was then measured. As Figure 2 illustrates, inhibition of insulin-simulated transport was observed within 5 minutes and was complete by 30 minutes. At this latter time, the morphology of the cells was unchanged and significant basal hexose transport activity was still observed (data not shown). These results suggest that wortmannin is inhibiting hexose transport by blocking PI 3-kinase activity and argue against the possibility that its inhibitory effects are due to general cytotoxicity.

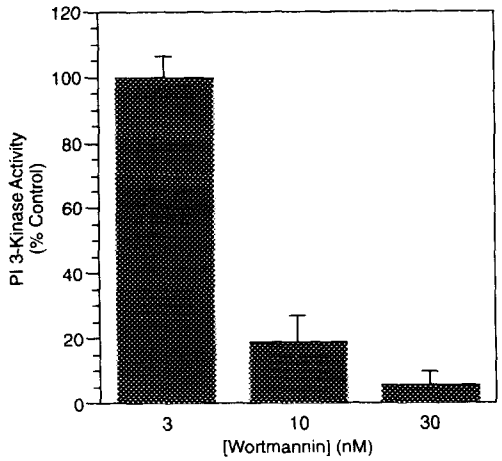


Figure 3. Inhibition by wortmannin of insulin-stimulated PI 3-kinase in L6 myotubes. Cells were incubated alone (control) or with the indicated concentrations of wortmannin for 30 min at 37 °C then stimulated with 100 nM insulin for 5 min at 37 °C . Lysates were prepared and solubilized proteins were immunoprecipitated with anti-phosphotyrosine antibodies conjugated to agarose beads. The PI 3-kinase activity in the immunoprecipitates was determined as described in the "Materials and Methods" section. Results presented are the means ± S.E. of triplicate determinations.

We have previously demonstrated that insulin activates PI 3-kinase activity approximately 2 to 3-fold in anti-phosphotyrosine immunoprecipitates of L6 myotubes (20). When insulin binds to its receptor the IRTK is activated and IRS-1 is phosphorylated. Presumably, the increased PI 3-kinase activity observed in anti-phosphotyrosine immunoprecipitates is the result of the increased interaction of tyrosine phosphorylated IRS-1 with the p85 regulatory subunit of PI 3-kinase. To examine the effects of wortmannin on insulin augmented PI 3-kinase activity, myotubes were acutely treated with insulin after being preincubated alone or with various concentrations of the compound. The cells were then washed and lysed, proteins were immunoprecipitated from cleared lysates with anti-phosphotyrosine antibodies and PI 3-kinase activity was assayed in the washed immunoprecipitates. As Figure 3 demonstrates, wortmannin inhibited PI 3-kinase activity in the stimulated cells at a dose as low as 10 nM; at 30 nM the enzyme was almost completely inhibited. Similar inhibition of PI 3-kinase activity by wortmannin has been reported in primary rat adipocytes and CHO/HIR cells (14, 21). It is concluded that PI 3-kinase is an element in the insulin pathway regulating glucose transport in L6 myotubes and that activation of PI 3-kinase is necessary for acute stimulation of transport by the hormone.

Vanadate, a phosphotyrosine phosphatase inhibitor, activates glucose and lipid anabolic metabolism in insulin-responsive tissue (22). In addition, oral administration of vanadate to streptozotocin-treated, hyperglycemic rats lowered

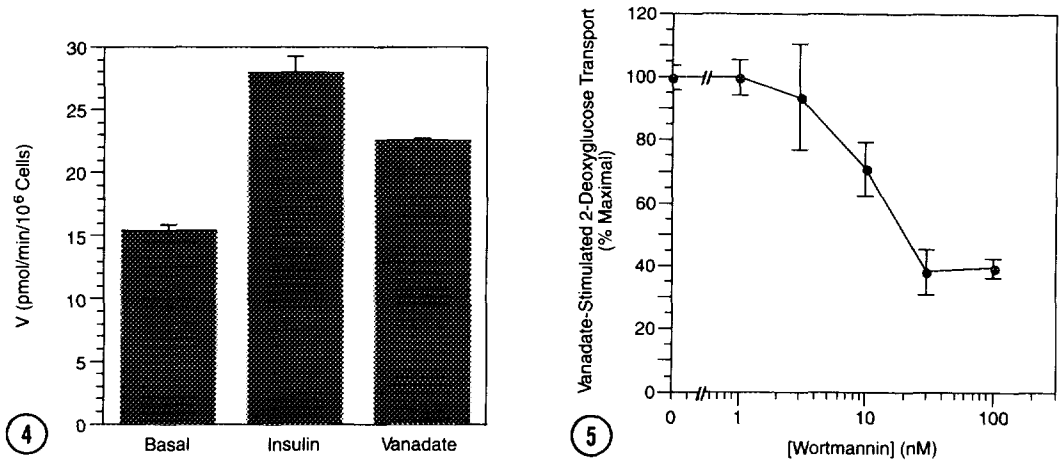


Figure 4. Stimulation by vanadate of 2-deoxy-D-[$^3$H]glucose transport into L6 myotubes. Cells were incubated alone or in the presence of 100 nM insulin or 10 mM vanadate for 30 min at 37 $^{o}$C. Transport of 2-deoxy-D-[$^3$H]glucose was measured as indicated in the "Materials and Methods" section. Transport in the presence of 40 μM cytochalasin B has been subtracted. Results presented are the means ± S.E. of triplicate determinations.

Figure 5. Effect of wortmannin on vanadate-stimulated 2-deoxy-D-[$^3$H]glucose transport into L6 myotubes. Cells were incubated for 30 min at 37 $^{o}$C with the indicated concentrations of wortmannin. The cells were then treated with 10 mM vanadate for an additional 30 min. Transport of 2-deoxy-D-[$^3$H]glucose was measured as indicated in the "Materials and Methods" section. Transport in the presence of 40 μM cytochalasin B has been subtracted. Results presented are the means ± S.E. of triplicate determinations.

blood glucose levels to normal (23). The effects of vanadate on the insulin pathway are complex, but it has been demonstrated that it acts, primarily, downstream of the insulin receptor (22). Here, we found that 10 mM vanadate acutely activated hexose transport into L6 myotubes to more than 50% of the maximal insulin-stimulated level (Figure 4). To examine the effects of wortmannin on vanadate stimulation of glucose transport, L6 cells were stimulated with vanadate after being preincubated alone or with increasing concentrations of wortmannin. As shown in Figure 5, vanadate stimulation of transport was inhibited in a dose-dependent manner by the compound. However, maximal inhibition was only ~60%. In contrast to insulin, vanadate appears to activate hexose transport by acting at sites that are within PI 3-kinase-dependent and independent pathways.

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