REDISTRIBUTION OF GLUT1 GLUCOSE TRANSPORTERS BY WORTMANNIN IN CLONE 9 CELLS

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SUMMARY

Glucose is transported across plasma membrane by glucose transporters (Glut1-4) that are expressed in a tissue specific manner. Glucose transport is controlled by different mechanisms including activation, translocation and expression of glucose transporters. Insulin stimulates glucose transport 5-15 fold in skeletal muscle and adipose tissue by increased translocation of Glut4 from its intracellular reservoirs to plasma membrane using intracellular signal transduction pathways including activation of phosphatidylinositol (PI) 3-kinase. It was shown that this enzyme has an important role in distribution of glucose transporters between cell surface and intracellular reservoirs. We aimed to test the effect of wortmannin, a highly specific inhibitor of PI 3-kinase, on translocation and redistribution of Glut1 gluco.e transporters in Clone 9 cells that expressed only Glut1 isoform. To verify the content of cell membrane glucose transporter, we transfected Clone 9 cells by Glut1 gene carrying myc sequence in it and used this transfected cell line that expressed myc epitope in extracellular first loop of Glut1. Incubation of transfected cells with 1 μM wortmannin for 1 hour decreased glucose uptake significantly ($p < 0.05$). To assess whether this decrease was due to redistribution of Glut1 glucose transporters between cell surface and intracellular stores, we employed western blot analysis of plasma membrane using anti-Glut1 antibodies and immunoflourocence staining of cells and plasma membrane sheets by anti-Glut1 and anti-myc antibodies. There was a significant decrease in densitometric analysis of the blots in western blot of plasma membrane and in the light intensity in immunoflouroscence staining of cells and plasma membrane in wortmannin-treated cells in comparison with untreated cells. We concluded that wortmannin caused the accumulation of glucose transporters in intracellular sites and a decrease of glucose uptake in basal conditions.

Key words: *Glucose transport, Glut1, Wortmannin*

ÖZET

Glukoz, hücre içerisine dokulara göre spesifik olarak eksprese olan glukoz taşıyıcıları (Glut1-4) tarafından taşınır. Glukoz transportu, glukoz taşıyıcılarının aktivasyonu, translokasyonu ve sentezini içeren farklı mekanizmalarla kontrol eidlir. İnsülin'in, yağ ve kas dokusundaki glukoz transportunu, hücre içerisindeki Glut4 glukoz taşıyıcılarının plazma membranına translokasyonunu sağlayarak, 5-15 kat oranında artırdığı saptanmıştır. Bu olayda, fosfatidilinozitol (PI) 3-kinaz'ın aktivasyonunu da içeren çeşitli sinyal iletim yollarının rol aldığı düşünülmektedir. PI 3-kinaz'ın, glukoz taşıyıcılarının hücre içi ve plazma membranı arasındaki dağılımında etkili olduğu bildirilmiştir. Bu çalışmada, özgün bir PI 3-kinaz inhibitörü olan wortmaninin, yalnızca Glut1 izoformunu sentezleyen Clone 9 (C9) hücrelerindeki glukoz transportu ve Glut1'in hücre içi dağılımı üzerindeki etkisinin incelenmesi amaçlandı. Hücre membranındaki Glut1 miktarını değişik yöntemlerle ölçebilmek için C9 hücreleri, içerisinde myc sekansını da taşıyan Glut1 geni ile transfekte edildi. Glut1'in ekstraselüler bölgesinde myc epitopunu eksprese eden, uygun transfekte hücre klonu seçildi ve çalışmalarda bu klon kullanıldı. Bir saatlik 1 μM wortmannin inkübasyonunun, bu hücrelerde glukoz transportunu anlamlı olarak ($p<0.05$) azalttığı belirlendi. Glukoz transportundaki bu azalmanın, glukoz taşıyıcısının hücre içerisine translokasyonuna bağlı olup olmadığını test etmek için, anti-Glut1 antikorları ile plazma membranında Western blot analizi, anti-Glut1 ve anti-myc antikorları ile intakt hücreler ve hücre membranı plaklarında immunofloresans boyama yöntemi gibi yöntemler kullanıldı. Wortmanin ile inkübe edilen hücrelerin hücre membranında yapılan Western blot analizinin dansitometrik ölçümünde ve immunofloresans boyama yöntemi sonucu yapılan ölçümlerde, Glut1 miktarında kontrole göre anlamlı azalma belirlendi. Güçlü bir PI 3-kinaz inhibitörü olan wortmaninin, Glut1'ın hücre membranından hücre içerisine translokasyonuna neden olduğu belirlendi.

Anahtar sözcükler: *Glukoz transportu, glut1, wortmannin*

Because glucose is a universal energy producing substrate, the regulation of its transport into cells is of fundamental importance in cellular homeostasis. Recent biochemical and molecular-biological evidence indicates that facilitated glucose transport is mediated by at least five homologous transporter isoforms (Gluts) that are differentially expressed in various tissues (1-3). These transporters are believed to possess a similar two and three dimensional membrane topography that includes 12 trans membrane helices. A widely distributed Glut isoform, Glut1, is expressed at high levels in rat brain, human erythrocytes, and placenta, and is present at lower levels in virtually all tissues. A fourth form of transporter, Glut4, is specifically expressed at high levels in tissues in which the rate of glucose transport is markedly augmented by insulin; such tissues include cardiac and skeletal muscle and adipose tissue (1,4). A large body of evidence has now accumulated demonstrating that exposure to insulin results in a translocation of transporters from as yet not fully defined intracellular sites to the plasma membrane. In addition, recent evidence indicates that under basal conditions Glut4 in adipocytes circulates rapidly (with a half time of 2 min) between the plasma membrane and a much larger pool of intracellular sites (the latter accounting for 94% of the total sites), and that insulin increases the rate of insertion and decreases the rate of removal of Glut4 from the plasma membrane (1,4,5). Recent biochemical data have improved our understanding of the signal transduction pathways leading to insulin-stimulated glucose transport and glucose transporter translocation. During the last two years, phosphatdylinositol kinase (PI3-kinase) has emerged as a critical step for such events in adipocytes. A highly specific inhibitor of PI3-kinase, wortmannin, inhibits the lipid and serine kinase activity of PI3-kinase in the nanomolar concentration range. Inhibition of PI3-kinase prevents the glucose transporter translocation to the cell surface by insulin and results in the accumulation of glucose transporters in the intracellular reservoirs leading to no increase of glucose transport by insulin in adipocytes and myoblasts (6-8). In this study, we used wortmannin to assess the role of PI3-kinase in Glut1 glucose carrier distribution and glucose transport activity in Clone 9 cells and transfected cells. In order to examine the mechanisms of Glut1 translocation, we employed a novel and sensitive method to measure directly the quantities of c-myc epitope-tagged Glut1 (Glut1myc) on the cell surface of transfected cells. Using this novel cell system, we demonstrated that PI 3-kinase plays a role in the signal transduction of Glut1 translocation. The high sensitivity of our method made feasible the detection of Glut1 on the cell surface following wortmannin incubation. Our results indicate that inhibition of PI3-kinase by wortmannin causes a decrease in glucose transport due to the redistribution of Glut1 transporters in Clone 9 cells.

MATERIALS AND METHODS

Materials:

Clone 9 cells were obtained from the American Type Culture Collection (Rockville, MD). Calf serum and Dulbecco's modified Eagle's medium

(DMEM) were purchased from GIBCO (Grand Island, NY). Nitrocellulose paper (BA-85) was obtained from Schleicher and Schuell (Keene, NH). Plastic culture dishes (35 and 100 mm) and glass coverslips (#22x22-2) were purchased from Corning Glass Works (Medfield, MA) and Fisher Scientific (Pittsburgh, PA), respectively. Standard chemical compounds, phloretin, cytochalasin B, geneticin, bovine serum albumin, dimethyl sulfoxide, phenylmethlysulfonilfluoride (PMSF), p-phenylenediaimine (PPDA), sodium azide and wortmannin were purchased from Sigma Chemical (St. Louis, MO). Phosphate-buffered saline lacking both calcium and magnesium was employed throughout (PBS). 3-O-methyl-D-[1-3H]glucose (3-OMG; 2.4 Ci/mmol) and Enhanced Chemi-Luminescence (ECL) kit were purchased from Amersham (Chicago, IL). pGX2 plasmid containing nucleotides encoding 14 amino-acids of human myc (epitope-tag) inserted between Ile56 and Pro57 in the first putative extracellular loop of Glut1 was generously provided by Dr. Ebina (9). pCDNA3 was obtained from Invitrogen (Carlsbad CA) and the protein assay reagent, from Bio-Rad (Hercules, CA). Rabbit polyclonal antiserum against the COOH-terminal 13-amino acid sequence of GLUT1 was purchased from Charles Rivers Pharmaservices (Southbridge, MA). Fluorescence-conjugated goat anti-rabbit IgG fraction was obtained from Cappel Research Products (Durham, NC). Vitrogen 100 (collagen; 3 mg/ml) was purchased from Collagen Biomaterials (Fremont, CA). Sulfo NHS-LC-LC-biotin and streptavidin-agarose were purchased from the Pierce Chemical Co. (Rockford, IL). Anti-myc antibody (mAb 9E10) was purchased from Oncogene Science (Cambridge, MA). Gel/Mount (mounting medium) was purchased from Biomeda (Foster City, CA).

Cell culture and transfection:

Clone 9 cells were maintained on plastic culture dishes in DMEM containing 5.6 mM D-glucose, supplemented with 10% calf serum at 37^0 C in 9% CO_2-humidified atmosphere (pH 7.4). For each experiment, cells were passaged at 1:8 dilution and seeded on 35- or 100-mm culture dishes in 2 or 8 ml of medium, respectively. All studies were carried out on replicate control and experimental culture plates in parallel. The medium was changed to DMEM devoid of serum 24 h before initiation of all experiments. Wortmannin (1 uM in DMEM) or diluent alone (DMEM) were added directly to the culture medium. Cells were co-transfected employing calcium phosphate-precipitation technique (10) with pGX2 plasmid (11), which contains the myc epitope in the first extracellular loop of Glut1, and pCDNA3 expressing the neomycin-resistance gene. Stable clones were selected employing G418 at 0.6 mg/ml. A clone containing ~3-fold higher amounts of Glut1 was chosen for further study. The transfected clone was maintained in a medium containing 0.125 mg/ml G418.

Measurement of 3H-3-OMG uptake:

Confluent cells on 35-mm culture plates in triplicate were used for the uptake measurements. The uptake was terminated after 1 minute of incubation. The accumulation of 3H-3-OMG at 1

min in either control or wortmannin incubated cells did not exceed 30% of its corresponding steady-state value. Cytochalasin B-inhibitable 3-OMG uptake was calculated as the difference between the uptake in each three plates in the absence of cytochalasin B and the mean uptake in the three plates in the presence of wortmannin, assayed in parallel.

Cell surface biotinylation and isolation of plasma membranes:

100-mm plates of confluent cells (3 plates per experimental conditions) were rinsed three times with 5 ml ice-cold PBS followed by incubation on a shaking platform for 30 min with 2 ml of ice-cold biotinylation buffer (120 mM NaCl, 30 mM NaHCO3, and 5 mM KCl; pH 8.5) containing 0.1 mg/ml freshly added sulfo-NHS-LC-LC biotin. Subsequent steps were as described with no modifications (12).

Western blot analysis:

SDS-PAGE was performed according to the method of Laemmli with the following modification: cell fractions containing equal amounts of protein (20 and 50 ug) were solubilized in Laemmli buffer containing 5% mercaptoethanol and incubated in 60^0 C water bath for 30 min. Solubilized protein samples were separated by polyacrylamide gel electrophoresis and electroblotted on the nitrocellulose paper according to the standard methods. Cell lysates were prepared employing a Nonidet P-40 containing buffer (13,14). After centrifugation to remove the nuclei, samples of supernatant were taken for analysis of protein and the remainder was mixed with 2x Laemmli buffer containing 10 mM dithiothreitol, frozen at -80^0 C and assayed within 1 week. Samples containing equal amounts of protein (20 ug) were denatured by heating for 30 min at 60^0 C, fractionated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels, and electroblotted according to standard methods. Blots were incubated with primary anti-Glut1 and anti-myc antibodies and with secondary rabbit serum containing polyclonal antibodies directed against the COOH-terminal 13-amino acid peptide of Glut1 (12) at 1:300 dilution or with a monoclonal antibody directed against 14 amino-acids of the c-myc epitope (9) at a concentration of 2.0 ug/ml. Secondary antibodies were employed at 1:2000 dilution and the blots were developed by the ECL kit.

Immunofluorescence staining and quantitation of fluorescence intensity

Incubation with anti-Glut1 antibodies:

Cells grown to ~75% confluence on glass coverslips were treated with diluent or azide for 1 h. Coverslips were rinsed briefly in ice-cold PBS and then fixed with methanol at -20^0 C for 5 minutes. After washing with PBS, cells were permeabilized with 0.5% Tween20 in PBS for 20 min at 25^0 C. Cells were then washed 3 times with PBS and reacted with rabbit anti-Glut1 antiserum at a 1:100 dilution in 0.2% goat serum in PBS for 1 h at 25^0 C. Coverslips were washed three times with PBS and incubated with fluorescein-conjugated goat anti-rabbit antibody at a 1:100 dilution in PBS for 1 h. Coverslips were then washed with 0.5% Tween20 in PBS three

times for 5 min each and mounted on glass slides with Gel/Mount fortified with 0.1% PPDA. Plasma membrane sheets were prepared by minor modification of previously described procedures (5). Cells were grown to 75% confluence on glass coverslips coated with collagen (0.2 mg/ml in 1.0 uM NaOH) and treated with wortmannin or diluent for 1 h. Coverslips were washed with PBS and then incubated for 10 sec in 1:4 dilution of buffer A (70 mM KCl, 30 mM Hepes, 5 mM MgCl2, 3 mM EGTA; pH 7.5), transferred to undiluted buffer A, and immediately sonicated twice employing an Heat-Systems Ultrasonics instrument (Farmingdale, NY) for 0.3 sec each at a setting of 20%. Plasma membrane sheets were fixed with 3% paraformaldehyde (in PBS) for 15 min at 25^0 C. After washing twice with PBS, coverslips were treated with anti-Glut1 antibody as described above.

Incubation with anti-myc antibodies:

Cells were grown and treated in diluent or wortmanin as described above. Coverslips were rinsed in ice-cold PBS and fixed with 3% paraformaldehyde in PBS for 15 min at 25^0 C. Coverslips were then washed with PBS three times and incubated with monoclonal anti-myc antibody at a 1:10 dilution in PBS (10 ug/ml) for 1 h at 25^0 C. After three washes with PBS, cells were incubated with fluorescein-conjugated goat anti-mouse antibody at 1:100 dilution in PBS for 1 h at 25^0 C. Coverslips were washed with 0.5% Tween20 in PBS three times for 5 min each and mounted on glass slides. Plasma membrane sheets were prepared and reacted with anti-myc antibody (10 ug/ml) as described above.

Photography and quantitation of fluorescence intensity:

In each experiment comparing two cell types or analyzing the effect of an agent such as wortmannin, the settings of the camera aperture and the time of exposure were kept constant. For quantitation of fluorescence intensity, eight areas from each coverslip were selected at random and the intensity (grey scale) was measured using Metamorphosis software (Worchester, PA) and a Gateway 2000 computer (Fryer Co., Huntlev, IL). The intensity values obtained from each coverslip were then averaged.

Immunoprecipitation:

Cells were metabolically labelled for 10 h in 0.10 mCi/ml ^{35}S-labeled sistein (ICN). Following the labelling, cells were lysed with lysis buffer. Cell lysates were immunoprecipitated with anti-myc antibodies. The samples were extensively washed, and resolved by 10 % SDS/PAGE electrophoresis followed by autoradiography.

Statistical analysis: All values are expressed as means $\pm$ SE, SD. In experiments involving two groups, Student's unpaired two-tailed t test was used to calculate p values and $p < 0.05$ was taken as significant. This study was conducted in the research laboratories of the Department of Medicine, CWRU, Cleveland, USA.

RESULTS

Firstly, we tested whether transfected cells expressed Glut1myc protein by immunoprecipitation using monoclonal anti-myc antibodies. Fig 1 shows that transfected cells express Glut1myc

protein. Next, we determined the effect of wortmannin on glucose transport. After incubation of transfected cells with 1 μM wortmannin for 1 hour, glucose transport decreased significantly ($p < 0.05$) (Fig 2). To assess whether this decrease was due to redistribution of Glut1 glucose transporters between cell surface and intracellular stores, we used the methods of western blot analysis of plasma membrane using anti-glut1 antibodies and immunoflourescence staining of cells and plasma membrane sheets by anti-Glut1 and anti-*myc* antibodies. In densitometric measurement of the blots (Fig 1) following western blot analysis of plasma membrane and in the measurement of light intensity of immunoflourescence staining of cells and plasma membrane (Fig 3 and Tables 1 and 2), there was a significant decrease in wortmannin-treated cells in comparison with untreated cells.

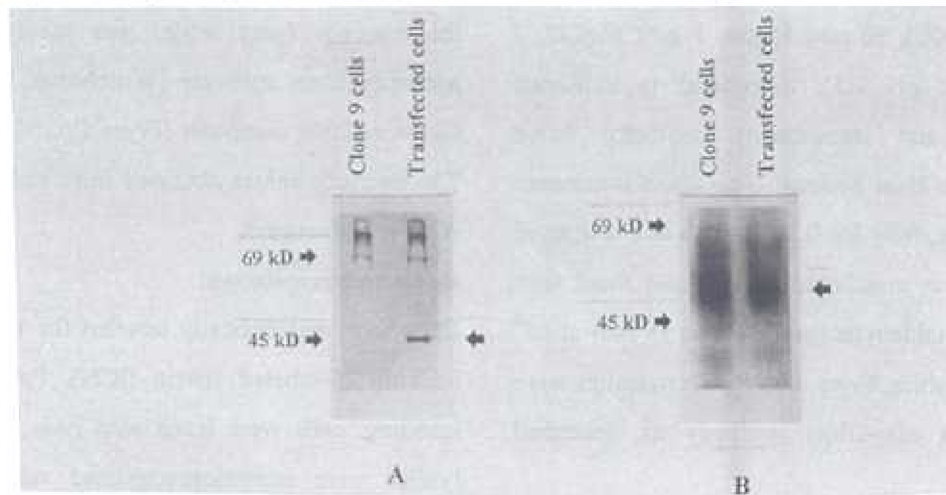

Figure 1. Immunoprecipitation and Western blot analysis showing plasma membrane Glut1 content of diluent and 1 μM wortmannin incubated cells for 1 hr.

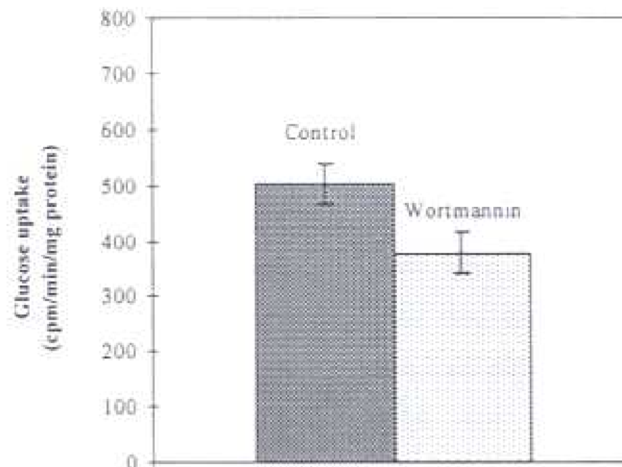

Figure 2. Glucose uptake after incubation with diluent (control) or 1 μM wortmannin for 1 hr in transfected cells.

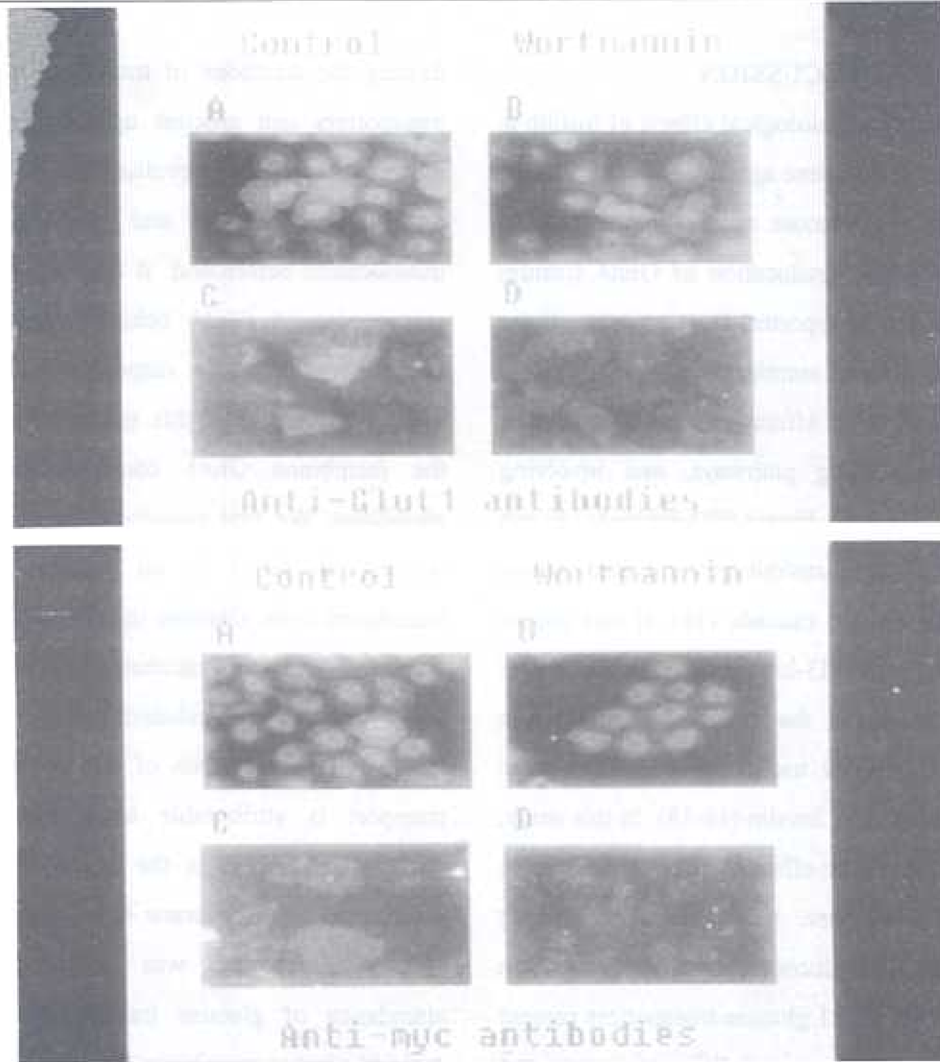

Figure 3. Immunoflourescence staining with anti-Glut1 (3a) and anti-myc (3b) antibodies in transfected cells incubated with diluent (control) and 1 μM wortmannin.

Table 1. The intensity of the light following the immunofluoroscence staining in control and wortmanin incubated intact cells.

	The mean intensities of light measured in intact cells (grey scale)	
	Anti-Glut1 antibodies	Anti-myc antibodies
Control	59 ± 1.9	57 ± 3.7
Wortmannin	53 ± 2.4	52 ± 2.7
p value	p<0.05	p<0.05

Table 2. The intensity of the light following the immunofluoroscence staining in control and membrane sheets of cells incubated with wortmannin.

	The mean intensities of light measured in membrane sheets (grey scale)	
	Anti-Glut1 antibodies	Anti-myc antibodies
Control	55 ± 2.3	48 ± 5.6
Wortmannin	42 ± 3.5	44 ± 2.7
p value	p<0.001	p<0.05

DISCUSSION

One of the major physiological effects of insulin is the stimulation of glucose uptake in the target cells. Insulin-stimulated glucose uptake in the cells is caused mainly by translocation of Glut4 (insulin sensitive glucose transporter) from an intracellular pool to the plasma membrane (4,8,15). Insulin initiates its biological effects by activating at least two major signalling pathways, one involving phosphatidylinositol 3-kinase (PI3-kinase) (15) and the other involving a ras/mitogen-activated protein kinase (MAP kinase) cascade (16). It was shown that inhibition of PI3-kinase in myoblasts and adipocytes prevented the stimulation of glucose transport and glucose transporter translocation to the cell membrane by insulin (16-18). In this study, we have analysed the effect of the highly specific inhibitor of PI3-kinase, wortmannin, on glucose transport and Glut1 glucose transporter distribution in Clone 9 cells. Glut1 glucose transporters present mostly in the membrane of different tissues and cell lines including Clone 9 cells, and they are responsible for basal glucose transport (1,2). In order to examine the mechanisms of Glut1 translocation, we employed a novel and sensitive method to measure directly the quantities of c-myc epitope-tagged Glut1 (Glut1myc) on the cell surface of transfected cells. We inserted synthetic oligonucleotids of the c-myc epitope (14 amino acids) into the first external loop of Glut1 cDNA. Glucose uptake by cells exceeded that of control cells. Our and another study showed that c-myc epitope insertion in the first external loop did not destroy the functions of translocation of glucose transporters and glucose uptake (9). The same technique was used to evaluate the effect of insulin on glucose transport and glucose transporters translocation before and it was reported that myc epitope tagged Glut4 behaves similarly to the endogenous Glut4 in response to insulin (17). Taking advantage of this system, we determined the membrane Glut1 content using anti-myc antibodies. We first examined the effect of 1 uM wortmannin for 1 hr on glucose transport in transfected cells. Glucose uptake was significantly lower in wortmannin incubated cells in comparison with the diluent incubated cells. To determine whether the mechanism of this early decrease of transport is attributable to a translocation of glucose transporters to the inside of the cell, we determined cell membrane Glut1 content. For this aim, western blotting was used to quantitate the abundance of glucose transporters in partially purified plasma membrane fractions isolated from the diluent and wortmannin treated cells. Rabbit polyclonal antibodies directed against the carboxy-terminal dodecapeptide of Glut1 were used in these studies. The relative abundance of Glut1 in plasma membrane extracts of cells incubated with wortmannin was significantly decreased in comparison with diluent treated cells. In addition, immunoflourescence staining of intact cells and membrane sheets using anti-Glut1 and anti-myc antibodies supported these findings (Fig. 3a and 3b). Preparation of membrane sheets has been used in many studies to detect the changes in membrane

proteins in literature taking advantage of the measuring of light intensity (5,19). We modified this preparation using collagen to fix the cells on the surface of the plate. In these experiments, a significant decrease in cell membrane Glut1 content was also determined. These findings show that the decrease of glucose uptake by wortmannin was mainly caused by translocation of Glut1 molecules to the intracellular pools. Another study also showed that wortmannin blocked the elevation of glucose transport in wild-type virus-infected mouse fibroblasts with no significant change in levels of either Glut1 mRNA or protein. It was asserted that the posttranslational mechanisms in the elevation of glucose transport in some conditions were the activation of PI 3-kinase and the translocation of Glut1 to the cell membrane (7). Our findings were compatible with these assertions and with the findings of other study which declared decreased plasma membrane Glut1 glucose transporters by wortmannin in myoblasts (6).

We concluded that wortmannin caused the accumulation of glucose transporters in intracellular sites and a decrease the glucose uptake in basal conditions.

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