Regulation of glucose transporters by insulin and extracellular glucose in C<sub>2</sub>C<sub>12</sub> myotubes

Taku Nedachi and Makoto Kanzaki

TUBERO/Tohoku University Biomedical Engineering Research Organization, Tohoku University, Sendai, Japan

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Nedachi, Taku, and Makoto Kanzaki. Regulation of glucose transporters by insulin and extracellular glucose in C<sub>2</sub>C<sub>12</sub> myotubes. *Am J Physiol Endocrinol Metab* 291: E817–E828, 2006. First published May 30, 2006; doi:10.1152/ajpendo.00194.2006.—It is well established that insulin stimulation of glucose uptake in skeletal muscle cells is mediated through translocation of GLUT4 from intracellular storage sites to the cell surface. However, the established skeletal muscle cell lines, with the exception of L6 myocytes, reportedly show minimal insulin-dependent glucose uptake and GLUT4 translocation. Using C<sub>2</sub>C<sub>12</sub> myocytes expressing exofacial-Myc-GLUT4-enhanced cyan fluorescent protein, we herein show that differentiated C<sub>2</sub>C<sub>12</sub> myotubes are equipped with basic GLUT4 translocation machinery that can be activated by insulin stimulation (~3-fold increase as assessed by anti-Myc antibody uptake and immunostaining assay). However, this insulin stimulation of GLUT4 translocation was difficult to demonstrate with a conventional 2-deoxyglucose uptake assay because of markedly elevated basal glucose uptake via other glucose transporter(s). Intriguingly, the basal glucose transport activity in C<sub>2</sub>C<sub>12</sub> myotubes appeared to be acutely suppressed within 5 min by preincubation with a pathophysiologically high level of extracellular glucose (25 mM). In contrast, this activity was augmented by acute glucose deprivation via an unidentified mechanism that is independent of GLUT4 translocation but is dependent on phosphatidylinositol 3-kinase activity. Taken together, these findings indicate that regulation of the facilitative glucose transport system in differentiated C<sub>2</sub>C<sub>12</sub> myotubes can be achieved through surprisingly glucose-dependent modulation of the activity of glucose transporter(s), which apparently contributes to obscuring the insulin augmentation of glucose uptake elicited by GLUT4 translocation.

We herein also describe several methods of monitoring insulin-dependent glucose uptake in C<sub>2</sub>C<sub>12</sub> myotubes and propose this cell line to be a useful model for analyzing GLUT4 translocation in skeletal muscle.

SKELETAL MUSCLE is one of the major insulin-target tissues responsible for the maintenance of whole body glucose homeostasis and accounts for the bulk of insulin-stimulated glucose disposal (70–80%) after a meal (9, 41). The maintenance of glucose homeostasis is critical for normal physiology, and alteration of blood glucose levels directly affects a variety of insulin actions. For example, chronic hyperglycemia, the predominant metabolic state of diabetes, can exacerbate defective glucose disposal by interfering with insulin action in insulin-target tissues, including skeletal muscle (7, 15). In skeletal muscle, insulin and exercise play central roles in the regulation of glucose transport and its metabolism.

Glucose transport in skeletal muscle is regulated by a facilitative glucose transport system involving at least two members of the glucose transporter family, GLUT1 and GLUT4 (39). GLUT1, which is essentially ubiquitous in its tissue distribution, is targeted predominantly to the plasma membrane and is therefore thought to mediate basal glucose transport in various cell types, including skeletal myocytes (38). On the other hand, GLUT4 is expressed at very high levels in skeletal muscle, cardiac muscle, and adipocytes, cell types that display the highest levels of insulin-stimulated glucose uptake (56). Under basal conditions, the GLUT4 isoform is sequestered to intracellular membrane compartments. In contrast, stimulation by insulin, muscle contraction, and/or hypoxia increases GLUT4 at the cell surface by promoting its translocation from intracellular GLUT4 storage compartments, allowing enhanced glucose transport in cells (55). This insulin-induced GLUT4 translocation accounts for a significant portion of insulin-stimulated glucose uptake, and defects in this insulin action in skeletal muscle contribute to the insulin-resistant characteristics of type 2 diabetes (25). Analyses of the mechanistic details of insulin- and exercise-induced glucose uptake in skeletal muscle are often performed using whole animal experiments, mainly because of the lack of available cell culture models that clearly and accurately reflect muscle glucose disposal in vivo. Therefore, despite the dominant role of skeletal muscle in normal physiology and the pathophysiology of type 2 diabetes (8), the precise molecular mechanism underlying GLUT4 translocation in response to these various stimuli in muscle cells remains to be determined.

Recently, substantial progress in understanding the insulin-induced GLUT4 translocation process has been made by using cultured adipocyte models (54). Both adipocytes and skeletal muscle cells exhibit insulin-stimulated GLUT4 translocation and glucose uptake that is completely dependent on phosphatidylinositol (PI) 3-kinase activity and therefore may employ a similar or identical mechanism in this regard. However, enhanced glucose transport in skeletal muscle cells is also induced by an insulin-independent mechanism that is activated by muscle contractions, and hypoxia (2, 40), thought to be mediated via the activation of 5′-AMP-activated protein kinase (AMP kinase) but not the PI 3-kinase signaling pathway (16, 30). Furthermore, recent studies suggested that AMP-kinase activation is not sufficient to explain the entire contraction-dependent glucose uptake (37), and the newly identified second pathway, Ca<sup>2+</sup>- and calmodulin-dependent protein kinase II pathway, is also involved in the contraction-dependent glucose uptake (58). In addition, adipocytes and muscle cells often respond differently to metabolic alterations. For example, under hyperglycemic conditions, basal glucose transport is de-
increased in skeletal muscle (21, 24, 40, 53) but not in adipocytes (5, 14). Thus a skeletal muscle cell line applicable to the study of GLUT4 translocation in response to insulin and other stimuli would be very useful for providing new insights into the muscle-specific regulatory mechanisms and novel proteins involved in this process.

To date, several cell lines derived from skeletal muscle (L6, C2C12, and BC3H1) have been used for studying insulin actions and glucose metabolism, including GLUT4 translocation (33, 34, 49). Among these skeletal muscle cell lines, the L6 line derived from rat skeletal muscle is the most frequently utilized as a cellular model system for investigating the insulin-stimulated glucose transport system (45). Both L6 myoblasts and myotubes appear to have GLUT4 translocation machinery that can be activated by insulin stimulation (50). Furthermore, GLUT4 overexpression results in increased insulin-induced glucose uptake regardless of whether or not these cells are differentiated, although the insulin action is considerably less than that in intact muscle (50). On the other hand, C2C12 cells derived from mouse skeletal muscle are mostly used to investigate muscle cell differentiation and development, including myofibrillogenesis, sarcomere development, and myotube contractions (6, 22, 35), whereas this cell line has rarely been used for studying insulin-dependent glucose transport. C2C12 cells do not express sufficient levels of GLUT4 proteins, and their insulin responsiveness is reportedly minimal even after differentiation (27, 46). Furthermore, previous reports have indicated that neither artificial overexpression nor dexamethasone-induced expression of GLUT4 augmented insulin-responsive glucose uptake (27, 49). Although the molecular basis underlying the differences between L6 and C2C12 cells remains unknown, C2C12 cells usually differentiate more efficiently than L6 cells in terms of the sarcomere formation that is required for muscle contractile activity (11, 32).

The present study was designed to determine whether insulin-responsive glucose transport machinery exists in C2C12 myotubes. Using C2C12 myotubes expressing exofacial myc-GLUT4-enhanced cyan fluorescent protein (ECFP), we observed that C2C12 myotubes exhibited significantly (~3-fold) increased GLUT4 translocation in response to insulin. However, this insulin stimulation of GLUT4 translocation did not reflect the net amount of 2-deoxyglucose uptake with a conventional assay protocol. We therefore attempted to identify the reason for the apparent discrepancy and found basal glucose uptake, most likely mediated through GLUT1, to be rapidly modulated by the extracellular glucose concentration, which obscures the insulin-responsive GLUT4-mediated augmentation of glucose uptake.

MATERIALS AND METHODS

Materials. 2-Deoxy-3H-glucose (37.2 Ci/mmol) was obtained from PerkinElmer Life and Analytical Science (Boston, MA). The Western blot detection kit (West superfermo detection reagents) was from Pierce Biotechnology (Rockford, IL). DMEM, penicillin/streptomycin, and Trypsin-EDTA were purchased from Sigma Chemicals (St. Louis, MO). Cell culture equipment was from BD Biosciences (San Jose, CA). Calf serum (CS) and FBS were obtained from BioWest (Nuaillé, France). Immobilon-P was from Millipore (Bedford, MA). Unless otherwise noted, all chemicals were of the purest grade available from Sigma Chemicals.

Cell culture. Mouse skeletal muscle cell lines, C2C12 myoblasts (59), were maintained in DMEM supplemented with 10% FBS, 30 µg/ml penicillin, and 100 µg/ml streptomycin (growth medium) at 37°C under a 5% CO2 atmosphere. For biochemical study, cells were grown on four-well plates (Nalgen Nunc International, Rochester, NY) at a density of 1 x 10³ cells/well in 5 ml of growth medium or on six-well plates (BD Biosciences) at a density of 3 x 10⁴ cells/well in 3 ml of growth medium. Three days after plating, cells had reached ~80–90% confluence (day 0). Differentiation was then induced by switching the growth medium to DMEM supplemented with 2% CS, 30 µg/ml penicillin, and 100 µg/ml streptomycin (differentiation medium). The differentiation medium was changed every 24 h. For the immunofluorescent staining study, cells were grown on 22-mm glass cover slips (model C022221; Matsunami, Osaka, Japan) in six-well plates.

Cloning of myc-GLUT4-ECFP-C2C12. A retroviral technique was used to obtain C2C12 clones stably expressing rat GLUT4, possessing the c-myc epitope tag in the first extracellular loop and ECFP at the carboxyl terminus. Briefly, established exofacial myc-GLUT4-EGFP (19) was replaced with ECFP and then inserted in a pBABE-puro retroviral vector (36). Next, pBABE-myc-GLUT4-ECFP was transfected in Plat E cells using Fugene 6 transfection reagents (Roche Applied Science, Indianapolis, IN), and high-titer retroviral supernatants were obtained. C2C12 myoblasts were infected with the generated retrovirus in growth medium containing 10 µg/ml of Polybrene. After infection (2 days), positive selection was performed in the presence of 5 µg/ml of puromycin, and 40 single clones were isolated. Forty individual clones were analyzed according to both myc-GLUT4-ECFP expression levels and the morphological parameter of myogenic differentiation, and a G47-C2C12 clone (GLUT4-clone 7-C2C12) expressing high levels of myc-GLUT4-ECFP was selected. Wild-type C2C12 (WT-C2C12) and G47-C2C12 clone were independently cultured until each experiment. Because all of these myoblast clones expressing myc-GLUT4-ECFP did not differentiate well by themselves, we took advantage of the characteristics that myoblasts fuse each other to form multinuclear myotubes. Namely, several clones expressing myc-GLUT4-ECFP appeared to differentiate well and fuse to form myotubes when they were cocultured with WT-C2C12 (Fig. 1). Thus WT-C2C12 were mixed with the GLUT4-C2C12 clone (G47-C2C12 + WT-C2C12 = 1:1) before each experiment so that we could obtain well-differentiated myotubes expressing an appropriate level of myc-GLUT4-ECFP. The myc-GLUT4-ECFP expression in G47-C2C12 cells and their differentiated myotubes was routinely confirmed by both Western blotting analysis and microscopic analysis using ECFP fluorescence or immunostaining with anti-c-myc antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA).

Western blot analysis. The expression and phosphorylation of each protein were analyzed by Western blot analysis. In brief, the harvested cell lysates were subjected to 5 or 12% SDS-PAGE (1:30 bis-acrylamide). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and the membranes were then blocked for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20. Immunostaining to detect each protein was achieved with a 1:10,000 dilution of anti-BD living colors for detecting ECFP (JL-8; BD Biosciences), anti-myogenin (F5D; GeneTex, San Antonio, TX), anti-integrin-β1 (BD Biosciences), anti-phosphospecific protein kinase B (Akt; Ser473; Cell Signaling Technology), or anti-phosphospecific extracellular signal-regulated kinase (ERK)5 antibodies that cross react with ERK1/2 (Thr202/Tyr204 for ERK1/2; Cell Signaling Technology). Specific total or phosphoproteins were visualized after subsequent incubation with a 1:5,000 dilution of anti-mouse or rabbit IgG conjugated to horseradish peroxidase and a SuperSignal Chemiluminescence detection procedure (Pierce Biotechnology). Protein concentrations were determined using a bichinonic acid assay (BCA; Pierce Biotechnology). Three independent experiments were
performed for each condition. Coomassie blue staining was also performed to assess the efficiency of protein transfer.

2-Deoxyglucose uptake assay. A 2-deoxyglucose uptake assay was performed as previously described (20). Briefly, after serum starvation for 4 h, C2C12 myotubes were washed with Krebs-Ringer phosphate-HEPES buffer [KRP buffer (in mM): 10 phosphate buffer, pH 7.4, 1 MgSO4, 1 CaCl2, 136 NaCl, 4.7 KCl, and 10 HEPES, pH 7.6] and then incubated without or with 100 nM insulin for 0–120 min in KRP buffer. Glucose transport was determined by the addition of 2-deoxy-[3H]glucose (0.1 mM, 0.5 μCi/ml; PerkinElmer Life and Analytical Science). After four min of incubation with KRP buffer containing 2-deoxy-[3H]glucose, the reaction was stopped by PBS with 10 μM cytochalasin B (Sigma), and the cells were washed three times with ice-cold PBS. The cells were then lysed in PBS containing 0.2 M NaOH, and glucose uptake was assessed by scintillation counting. Cytochalasin B (20 μM) was added to the assay buffer for the measurement of nonspecific background. Results are specific uptake, i.e., the background subtracted from the total uptake, expressed as the mean ± SE of the indicated number of experiments. The protein content was determined in each experiment with a BCA protein assay kit (Pierce Biotechnology). For each experiment, at least two assays of each condition were performed, and each experiment was repeated at least three times.

Anti c-myc antibody uptake assay. For the measurement of insulin-dependent GLUT4 translocation, the C2C12 myotubes expressing myc-GLUT4-ECFP were serum starved for 4 h, washed three times with KRP buffer, and then placed in a CO2 incubator with 2 ml of KRP buffer in the presence or absence of 200 nM wortmannin. After 10 min of incubation, 4 μg/ml of the anti-c-myc antibody were added to the buffer, and the cells were stimulated with or without 100 nM insulin for 60 min. In some cases, 15 min after insulin addition, D(+)-glucose was added to the KRP buffer. For evaluating the effects of extracellular glucose on myc-GLUT4-ECFP translocation, the myc-GLUT4-ECFP-C2C12 myotubes were incubated with serum-free DMEM containing 0, 5, or 25 mM glucose for 3 h. Next, 4 μg/ml of the anti-c-myc antibody were added to the medium. After an additional 1-h incubation with the anti-myc antibody, the cells were placed on ice to stop the reaction and washed three times with PBS. Next, the cells were harvested using 1× Laemml’s buffer and subjected to Western blot analysis using anti-mouse IgG antibody, anti-c-myc antibody, or anti-BD living color antibody (JL-8; BD Biosciences).

Immunofluorescent analysis. WT-C2C12 and G47-C2C12 myoblasts were mixed at a 1:1 ratio on glass cover slips placed in six-well plates. After 6 days of differentiation, the serum-starved cells were stimulated with 100 nM of insulin for 60 min. The D-medium was changed every 24 h. D: after 6 days, cell lysates were prepared, and myogenic marker proteins were analyzed by Western blotting analysis as described in MATERIALS AND METHODS.

RESULTS

We established C2C12 myotubes expressing a minimal, but readily detectable, level of exofacial myc-GLUT4-ECFP (G47-C2C12), as described in MATERIALS AND METHODS, to evaluate whether GLUT4 translocation machinery is conserved in C2C12 myotubes. Tubule formation in C2C12 myocytes did not differ between WT-C2C12 alone and a mixture of WT-C2C12 and G47-C2C12 clone (Fig. 1, A–C). In addition, expressions of myogenic differentiation markers such as myogenin were similar in the differentiated WT-C2C12 alone and the blended
Given that >95% of myotubes were ECFP positive, most of the G47-C2C12 myoblasts underwent differentiation, fusing with WT-C2C12. Using the G47-C2C12 myotubes, we first examined the effects of insulin stimulation on net glucose transport activity as assessed by a conventional 2-deoxy-[3H]glucose uptake assay using glucose-free KRPH buffer (20). Unlike L6 myocytes, previously reported to display enhanced insulin-dependent glucose uptake (∼2- to 6-fold) with artificial expression of exogenous GLUT4 (44, 45), G47-C2C12 myotubes showed a significant (P < 0.01) but far smaller increase in glucose uptake in response to insulin stimulation (∼1.3- to 1.4-fold; Fig. 2A). The total amount of 2-deoxy-[3H]glucose uptake in the basal state and the increment with insulin stimulation were very similar to those in WT-C2C12 myotubes, indicating the level of myc-GLUT4-ECFP expression in G47-C2C12 myotubes to be minimal (Fig. 2A). To evaluate GLUT4 translocation in G47-C2C12 myotubes employing the myc-GLUT4-ECFP as a reporter, we next performed an immunocytochemical study using the anti-myc antibody (Myc Ab) (Fig. 2B). Nonpermeabilized G47-C2C12 myotubes were reacted with Myc Ab, which was subsequently detected by rhodamine-conjugated rabbit anti-mouse IgG such that we were able to detect only myc-GLUT4-ECFP fully incorporated in the plasma membrane. Under basal conditions, G47-C2C12 myotubes displayed no detectable surface expression of myc-GLUT4-ECFP as assessed by Myc Ab immunostaining (Fig. 2Bb), and most of the myc-GLUT4-ECFP was localized to intracellular compartments as assessed by ECFP fluorescence (Fig. 2Bd). In contrast, after insulin stimulation for 1 h, G47-C2C12 myotubes displayed marked cell-surface expression of myc-GLUT4-ECFP detectable by Myc Ab (Fig. 2Be), although the majority of myc-GLUT4-ECFP remained intracellular and the apparent localization of myc-GLUT4-ECFP at the plasma membrane was difficult to detect by ECFP fluorescence (Fig. 2Dd). We also quantitated these data by counting the number of surface Myc-positive myotubes among ECFP-positive myotubes (defined as multinuclear myotubes that contained >5 nuclei; Fig. 2C). In the basal state, GLUT4 translocation as assessed by counting myc-positive cells was observed in ∼30% of myotubes. In contrast, insulin-induced

![Graph](image.png)

**Fig. 2.** Effects of insulin on total 2-deoxyglucose uptake and GLUT4 translocation to plasma membrane. C2C12 clone stably expressing exofacial myc-GLUT4-ECFP was isolated as described in MATERIALS AND METHODS. WT-C2C12 and G47-C2C12 were mixed at a ratio of 1:1 (WT-C2C12-G47-C2C12), and differentiation in multinuclear myotubes was promoted by incubation in DMEM + 2% CS for 6 days. A: differentiated C2C12 myotubes expressing myc-GLUT4-ECFP (G47) or WT-C2C12 myotubes (wild type) were serum starved for 4 h with low-glucose DMEM (LG-DMEM) followed by preculture with glucose-free Krebs-Ringer phosphate-HEPES (KRPH) buffer for 10 min and then stimulated with or without 100 nM insulin for 60 min. After insulin treatment, the cells were placed on ice and washed three times with ice-cold KRPH, and 2-deoxy-[3H]glucose uptake was measured as described in MATERIALS AND METHODS. Values are presented as means ± SE. Open bars, basal; filled bars, insulin stimulation. *P < 0.01. B and C: myotubes were serum starved for 4 h with LG-DMEM followed by 100 nM insulin stimulation for 60 min. After insulin stimulation, the cells were fixed and subjected to immunocytochemical analysis using anti-c-Myc antibodies. Fluorescent signals were detected under confocal microscopy. Data were quantified (C) by counting the membrane surface myc positive myotubes (B) among ECFP (CFP)-positive myotubes (defined as multinuclear myotubes that contained >5 nuclei). Three independent experiments were performed, and >50 myotubes were counted in each experiment. Values are presented as means ± SE. Open bars, basal; filled bars, insulin stimulation. **P < 0.001.
GLUT4 translocation was observed in ~90% of myotubes. Although the amount of translocated GLUT4 in these myotubes was not large, there was enough to allow detection by the Myc Ab immunostaining method (~3-fold increase). Surface expression of myc-GLUT4-ECFP in response to insulin stimulation was completely inhibited by wortmannin, a PI 3-kinase inhibitor (data not shown). Taken together, these data strongly suggest each C2C12 myotube to be endowed with the basic GLUT4 translocation machinery needed to respond to insulin stimulation, although the fraction of GLUT4 proteins responsive to insulin was smaller than in 3T3-L1 adipocytes.

To confirm that insulin faithfully stimulated myc-GLUT4-ECFP translocation and its appearance at the plasma membrane in G47-C2C12 myotubes, we performed a continuous Myc Ab uptake assay allowing detailed quantitative analysis. The serum-starved G47-C2C12 myotubes were incubated for 1 h in glucose-free KRPH buffer containing 4 µg/ml of Myc Ab in the absence or presence of 100 nM of insulin, and the amount of antibody taken up was evaluated by immunoblotting (Fig. 3). A nonrelevant antibody was used at the same concentration to determine nonspecific binding/incorporation (Fig. 3A, top, lanes 4–6). Under these incubation conditions, a small amount of Myc Ab was associated with G47-C2C12 in the absence of insulin (Fig. 3A, top, lane 1), whereas an approximately three-fold increase in the uptake of Myc Ab was observed with insulin stimulation (lane 2), and this effect of insulin was completely abolished by wortmannin (lane 3). These data demonstrate that insulin-dependent GLUT4 translocation machinery exists in C2C12 myotubes (Figs. 2B and 3), although it is apparently not yet fully developed. Our data also clearly demonstrate that apparent discrepancy between total glucose uptake and myc-GLUT4-ECFP translocation induced by insulin in G47-C2C12 myotubes and that insulin stimulation of GLUT4 translocation is barely detectable with the conventional 2-deoxy-[^3]H]glucose uptake assay under these conditions (Fig. 2A).

It is well established that insulin stimulation of GLUT4 translocation accounts for a significant portion of the enhanced glucose uptake elicited by insulin in skeletal muscle (55). However, cultured myocytes usually exhibit far less augmentation of glucose uptake in response to insulin stimulation. One possible explanation for this phenomenon is that a high level of basal glucose uptake, most likely mediated through GLUT1 (38), may mask insulin-induced augmentation of glucose uptake in response to GLUT4 translocation. In addition, numerous reports have shown that extracellular glucose concentrations during the preincubation period affect basal glucose transport activity (18, 26, 47, 51, 57). To examine this possibility, we examined the regulatory properties of glucose transport activity according to changes in extracellular glucose levels in G47-C2C12 myotubes. Serum-starved G47-C2C12 myotubes were preincubated with KRPH buffer containing 0 or 25 mM glucose for 5, 15, 30, 60, or 120 min, and the basal glucose uptake was then measured by 2-deoxy-[^3]H]glucose uptake (Fig. 4A). After deprivation or administration of glucose for the indicated preincubation period, the cells were immediately placed on ice, washed with ice-cold glucose-free KRPH buffer three times, and then subjected to the 2-deoxyglucose uptake assay. As shown in Fig. 4A, we observed surprisingly rapid changes in extracellular glucose that affected basal glucose uptake. Glucose deprivation (5–15 min) promptly caused ~1.3- to 1.4-fold increases in basal glucose uptake, whereas an acute high-glucose (25 mM) challenge markedly decreased basal glucose uptake within 5 min. Although insulin stimulated an ~1.3- to 1.4-fold increase in glucose uptake as indicated in Fig. 2A, time course experiments revealed the stimulatory effect of insulin to be exerted considerably more slowly, and no significant augmentation of 2-deoxy-[^3]H]glucose uptake was detected after 5–15 min of insulin stimulation (Fig. 4B).

To ascertain whether the reduction of 2-deoxy-[^3]H]glucose uptake by preincubation with 25 mM glucose was the result of competition between remaining glucose and 2-deoxy-[^3]H]glu-
buffer. 2-Deoxyglucose uptake was measured as described in MATERIALS AND METHODS on ice to stop metabolic reactions and washed 3 times with ice-cold KRPH containing either 0 or 25 mM glucose. At the indicated time, cells were placed glucose (Glc) for 4 h, and the medium was switched to KRPH buffer myc-GLUT4-ECFP were serum starved with LG-DMEM containing 5 mM glucose (Glc) for 4 h, and the medium was switched to KRPH buffer containing either 0 or 25 mM glucose. At the indicated time, cells were placed on ice to stop metabolic reactions and washed 3 times with ice-cold KRPH buffer. 2-Deoxyglucose uptake was measured as described in MATERIALS AND METHODS.

Fig. 4. Time-dependent changes in 2-deoxyglucose uptake in the presence or absence of extracellular glucose. A: differentiated C2C12 myotubes expressing myc-GLUT4-ECFP were serum starved with LG-DMEM containing 5 mM glucose (Glc) for 4 h, and the medium was switched to KRPH buffer containing either 0 or 25 mM glucose. At the indicated time, cells were placed on ice to stop metabolic reactions and washed 3 times with ice-cold KRPH buffer. 2-Deoxyglucose uptake was measured as described in MATERIALS AND METHODS. B: differentiated C2C12 myotubes expressing myc-GLUT4-ECFP were serum starved with LG-DMEM containing 5 mM glucose for 4 h and then stimulated with 100 nM insulin for the indicated times. Again, a 2-deoxy-[3H]glucose uptake assay was performed.

cose, we measured 2-deoxy-[3H]glucose uptake after three different washing schedules (1, 3, or 6 times; Fig. 5A). Regardless of the number of times the preparation was washed, preincubation of G47-C2C12 myotubes in 25 mM glucose resulted in an ~50% decrease in 2-deoxy-[3H]glucose uptake. Importantly, the inhibitory effect of high-glucose administration on basal glucose uptake was not observed when the cells were preincubated with glucose on ice, indicating thermosensitive processes (e.g., enzyme activity, metabolic reactions) to be involved in this phenomenon (Fig. 5B). Moreover, this reduction in 2-deoxy-[3H]glucose uptake in response to a high extracellular glucose concentration was not attributable to changes in osmotic pressure, since neither mannitol nor sucrose, used as an osmotic replacement, produced this effect (Fig. 5C). Taken together, these data show that brief exposure of cultured myotubes to pathophysiologically high levels of glucose (25 mM) rapidly decreases glucose uptake, whereas glucose deprivation acutely increases glucose uptake. The data from our time course experiments also suggest that the regulatory mechanisms of glucose uptake modulated by extracellular glucose concentrations are different from those modulated by insulin.

To explore the mechanisms underlying the acute effects of extracellular glucose on glucose transport systems in C2C12 myotubes, we examined insulin stimulation of 2-deoxy-[3H]glucose uptake (net glucose transport activity) and Myc Ab uptake (total myc-GLUT4-ECFP translocation) in the presence or absence of glucose. When serum-starved G47-C2C12 myotubes were incubated with 100 nM insulin in the presence of 0, 5, or 25 mM glucose for 1 h (experimental design shown in Fig. 6A), the insulin-induced augmentation of glucose uptake was severely inhibited by pretreatment with 25 mM glucose (Fig. 6B), as well as insulin stimulation of protein kinase B/Akt phosphorylation (Ser473) and ERK1/2 phosphorylation (Thr202/Tyr204, Fig. 6C). However, when 25 mM glucose was administered at 15 min after insulin stimulation (experimental design shown in Fig. 7A), the subsequent enhancement of 2-deoxy-[3H]glucose uptake by insulin was preserved (Fig. 7B). The modulation of glucose uptake by extracellular glucose was not the result of exogenous expression of myc-GLUT4-ECFP since essentially the same results were obtained in WT-C2C12 myotubes (Fig. 7E). Importantly, the insulin dose-response curve of 2-deoxy-[3H]glucose uptake was not affected; only the net amount taken up was suppressed, in accordance with high-glucose treatments (Fig. 7D).

Taken together, these data clearly demonstrate that, in C2C12 myotubes, basal glucose transport activity is rapidly suppressed by preincubation with glucose (Figs. 6B and 7B). This is not because of the redistribution of GLUT4 protein (Figs. 6C and 7C, top, lanes 1, 3, and 5) but rather is most likely mediated through an alteration of the glucose transport activity of glucose transporter proteins (GLUT1/GLUT3) via modulation of their intrinsic activity and/or redistributions. To further characterize the glucose-dependent regulation of glucose transport activity, G47-C2C12 myotubes were treated with various inhibitors that have been shown to be involved in glucose transport systems, and 2-deoxy-[3H]glucose uptake was measured using assay protocol B (Fig. 7B). Under basal conditions, wortmannin, an inhibitor of PI 3-kinase, significantly inhibited the basal 2-deoxy-[3H]glucose uptake in glucose-free KRPH buffer, but this inhibitory effect was not observed in the presence of 25 mM glucose (Fig. 8A). Because glucose deprivation (preincubation in glucose-free KRPH buffer) rapidly increases basal 2-deoxy-[3H]glucose uptake as indicated in Fig. 3A, these results indicate that PI 3-kinase activity is required for the glucose deprivation-induced increase in basal 2-deoxy-[3H]glucose uptake. The glucose uptake modulated by glucose deprivation was specifically inhibited by wortmannin, but not by either SB-203580, an inhibitor of p38 mitogen-activated protein kinase (Fig. 8B) previously reported to be involved in the regulation of glucose transport activity (3, 43), or rapamycin, which inhibits mammalian target of rapamycin that can be activated by glucose administration (31).
DISCUSSION

The present studies were designed to clarify whether the C2C12 skeletal muscle cell line possesses the basic machinery required for translocation of GLUT4 in response to insulin stimulation. By using G47-C2C12 myotubes expressing exofacial Myc-GLUT4-ECFP, we clearly demonstrated that C2C12 myotubes are equipped with GLUT4 translocation machinery that can be activated by insulin stimulation (~3-fold; Figs. 2 and 3). Furthermore, our data document that the apparent discrepancy between 2-deoxy-[3H]glucose uptake and GLUT4 translocation induced by insulin in C2C12 myotubes results from acute autoregulation of glucose transporter(s), possibly already present at the plasma membrane, by extracellular glucose concentrations (Fig. 4). By exploiting the glucose-induced autoregulatory property of this glucose transport system, we were able to observe approximately twofold increases in the insulin-induced 2-deoxy-[3H]glucose uptake in G47-C2C12 myotubes (Fig. 7). In addition, it is noteworthy that a high level of glucose uptake was suppressed by wortmannin (Fig. 8), suggesting possible involvement of PI 3-kinase activity in the extracellular glucose-dependent autoregulatory mechanism by which the facilitative glucose transport system operates in C2C12 myotubes. Taken together, these findings provide compelling evidence that differentiated C2C12 myotubes contain basic GLUT4 translocation machinery that can be activated by insulin.

The C2C12 cell line is reportedly not a suitable model for investigating insulin stimulation of GLUT4 translocation, since these cells do not express sufficient levels of GLUT4 protein and their insulin responses are barely detectable with a conventional 2-deoxy-[3H]glucose uptake assay (27, 49). However, we observed an approximately twofold increase in 2-deoxy-[3H]glucose uptake with insulin, but only when extracellular glucose levels were properly controlled. This observation was further confirmed by the Myc Ab uptake assay and immunostaining analysis using G47-C2C12 myotubes expressing myc-GLUT4-ECFP. As we mentioned, our efforts to isolate a C2C12 clone expressing myc-GLUT4-ECFP that still possesses the ability to differentiate into myotubes failed. However, we established a new method to obtain well-differentiated myotubes expressing myc-GLUT4-ECFP by blending the wild type and the clone. Our method using the characteristics of myotubes, fusing myoblasts resulting in multinuclear myotubes, has several advantages. First, the expression levels of...
myc-GLUT4-ECFP in myotubes could be controlled by changing the mixing ratio of wild type and G47-C2C12 cells. Second, this method also allowed us to introduce another gene(s) of interest, in addition to myc-GLUT4-ECFP, by simply mixing the C2C12 clone that expressed those genes.

Tortorella and Pilch (49) reported the lack of an insulin-responsive vesicular compartment in dexamethasone-treated C2C12 myotubes expressing large amounts of endogenous GLUT4 protein, since they were unable to detect any augmentation of 2-deoxy-[^3H]glucose uptake with insulin stimulation. They also used membrane fractionation and surface biotinylation techniques to elucidate the redistribution of GLUT4 protein in response to insulin, but these methods were not applied successfully for this purpose. In our study using G47-C2C12 myotubes expressing exofacial myc-GLUT4-ECFP, even though surface expression of the c-myc tag was obviously increased by insulin stimulation, most ECFP signals were still detected inside the cells even after insulin stimulation (Fig. 2B). Thus, in C2C12 myotubes, only a small portion of GLUT4 can be translocated by insulin, i.e., much less than with skeletal muscle in vivo (>50% of GLUT4 translocates to the plasma membrane in response to insulin; see Refs. 60 and 61). This might be the reason for GLUT4 translocation being difficult to detect by fractionation analysis in cultured myotubes. In addition, the amount of 2-deoxy[^3H]glucose transported in the cells reflects the activities of all transporters expressed in the cells and therefore does not accurately reflect insulin-induced GLUT4 translocation. C2C12 myotubes, which express a large amount of GLUT1, thus exhibit very high basal glucose uptake levels that probably contribute to masking the insulin stimulation of glucose uptake achieved by GLUT4 translocation. In this regard, the Myc Ab uptake assay measures only GLUT4 protein exposed to the cell surface during a certain incubation period, possibly making it a very useful system for evaluating GLUT4 translocation without consideration of other glucose transporters such as GLUT1. Indeed, insulin stimulation resulted in an approximately threefold increase in the uptake of Myc Ab even in the absence of extracellular glucose (Figs. 6 and 7), a condition that produces higher basal glucose uptake (Fig. 4A). This result was further confirmed by an immunofluorescent staining study of the surface-exposed myc-GLUT4-ECFP in myotubes.
ECFP (Fig. 2C). In addition, detection of the cell surface-exposed GLUT4 by Myc Ab can pinpoint only GLUT4 fully incorporated in the plasma membrane, allowing GLUT4 proteins docked just beneath the plasma membrane to be distinguished from those that are completely fused.

A striking result of the present studies was the surprisingly rapid (within 5 min) effect of extracellular glucose on subsequent glucose uptake in C2C12 myotubes (Fig. 4A) that was not mediated via redistribution of GLUT4 (Fig. 6, C and F). In a conventional 2-deoxy-[^3H]glucose uptake assay protocol used for 3T3-L1 adipocytes (20), the cells were preincubated in glucose-free KRPH buffer for 10 min, and insulin was then added to the assay buffer to evaluate insulin stimulation of glucose uptake. Under these assay conditions, however, basal glucose uptake was rapidly increased in C2C12 myotubes (Fig. 4A), which is apparently an intrusive condition for measuring GLUT4-mediated glucose uptake. Numerous reports have demonstrated that glucose deprivation augments basal glucose uptake, whereas preexposure to a high concentration of glucose dose-dependently diminishes its uptake in various cell types, including skeletal muscle (47, 57). In addition, GLUT4 redistribution in response to hyperglycemia within 40 min was also observed in skeletal muscle of experimental diabetic animals (13). However, most studies using cultured muscle cells focused on relatively long (0.5-2 h) or chronic effects of extracellular glucose levels on the glucose transport system and indicated that glucose-dependent regulation of glucose transporter function involves complex mechanisms, including increased GLUT1 mRNA transcription and glucose transporter synthesis as well as changes in the subcellular distribution of glucose transporter proteins (23, 51, 52). We attempted to detect GLUT1 redistribution in response to extracellular glu-
cose levels by immunofluorescent staining but found no evidence of changes in its subcellular redistribution (data not shown). Thus the most plausible explanation for the glucose-dependent rapid changes in glucose transport activity, despite no obvious redistribution of GLUT4 protein, is a modulation of the intrinsic activity of GLUT1 already present at the plasma membrane, although we cannot rule out the possibility that extracellular glucose induced redistribution of an undetectable amount of GLUT1 within 5 min. Another important finding reported herein is that wortmannin significantly inhibited basal glucose uptake (Fig. 8A). Although little difference in basal phosphorylation levels of Akt (Ser473) and glycogen synthase kinase-3β (Ser21/22) was detected with glucose deprivation (data not shown), the evidence that wortmannin suppressed basal glucose transport activity suggests that basal levels of PI 3-kinase activity might be sufficient for exerting this effect.

In addition, our present data clearly demonstrate that acute autoregulation of glucose uptake by high glucose administration is dependent on a mechanism involving thermosensitive reaction(s), since the effect of high glucose administration, i.e., decreasing basal glucose uptake, was not observed at low temperatures (on ice; Fig. 5). Consistent with this idea, the intrinsic transport activity of GLUT1 has been reported to be modulated by glucose metabolites such as glucose 6-phosphate, a product catalyzed by hexokinase II (12, 48), and cytoplasmic ATP (4, 10, 17). Thus it is possible that glucose 6-phosphate and/or ATP catalyzed from the incorporated glucose in C2C12 myotubes contributes to modulating basal transport activity. On the other hand, administration of a high glucose concentration under assay protocol B did not alter insulin-induced GLUT4 translocation or 2-deoxy-[3H]glucose uptake (Fig. 7). Further studies are clearly required to identify the mechanism by which extracellular glucose differentially modulates the glucose transport system in C2C12 myotubes.

Fig. 8. Effects of wortmannin, rapamycin, and SB-203580 on extracellular glucose-controlled 2-deoxyglucose uptake in C2C12 myotubes. A: C2C12 myotubes expressing exofacial myc-GLUT4-ECFP were serum starved with LG-DMEM for 4 h. The cells were then stimulated with or without 100 nM insulin in the presence or absence of 100 nM wortmannin. After insulin stimulation (15 min), 0 or 25 mM glucose was added to the KRPH buffer (the same as in protocol B, shown in Fig. 7A). Sixty minutes after insulin stimulation (45 min after glucose addition), the cells were placed on ice and washed 3 times with ice-cold KRPH buffer, and 2-deoxy-[3H]glucose uptake was measured as described in MATERIALS AND METHODS. B: differentiated C2C12 myotubes expressing exofacial myc-GLUT4-ECFP were serum starved with LG-DMEM for 4 h. The cells were then incubated with KRPH buffer containing 0 or 25 mM glucose for 60 min in the presence or absence of 100 nM wortmannin or 10 μM SB-203580. A 2-deoxy-[3H]glucose uptake assay was performed.

Fig. 9. Schematic depiction of components of the facilitated glucose transport system in C2C12 myotubes. A: extracellular glucose deprivation enhanced glucose transport activity independently of GLUT4 translocation to the plasma membrane, possibly via activation of the intrinsic transport activity of GLUT(s) already present at the plasma membrane. This signal is dependent on phosphatidylinositol (PI) 3-kinase activity. B: hypothetical depiction of glucose uptake in C2C12 myotubes. Glucose uptake mechanisms in C2C12 myotubes could be classified into at least three mechanisms, as follows: 1) insulin-dependent glucose uptake, which is dependent on PI 3-kinase activity and GLUT4 translocation; 2) extracellular glucose-regulated glucose uptake, which is dependent on PI 3-kinase activity but is independent of GLUT4 translocation, and 3) basal glucose uptake, which is abolished by cytochalasin B, but is not dependent on extracellular glucose, PI-3 kinase activity, or GLUT4 translocation.
We also observed inhibitory effects of a high glucose concentration on Akt and Erk phosphorylations induced by insulin, as previously reported (Fig. 6D and Refs. 29 and 42). Impaired insulin signaling cascades may directly account for the disappearance of insulin-responsive GLUT4 translocation. Thus pathophysiologically high levels of glucose affect at least two distinct events as follows: one is the basal glucose uptake and the other is insulin receptor signaling. Importantly, however, the present experiments suggest that, once insulin signaling cascades are sufficiently triggered and functioning in cells in the absence of glucose, the subsequent high-glucose challenge effectively suppresses only the high basal glucose uptake while having no impact on insulin-induced GLUT4 translocation processes (and probably intrinsic transport activity). As a result, an approximately twofold increase in the insulin stimulation of 2-deoxy-[3H]glucose uptake was observed using assay protocol B (Fig. 7B).

Activation of AMP-kinase in skeletal muscle by exercise, hypoxia, glucose deprivation, or 5-aminoimidazole-4-carboxamide ribonucleoside has been implicated in the enhanced GLUT1-mediated glucose uptake mediated mainly through GLUT4 translocation (28). However, rapid modification of glucose uptake in response to changes in the glucose concentration (5 min) was not accompanied by changes in AMP kinase phosphorylation (Thr172; LKB phosphorylation site) or acetyl-CoA carboxylase phosphorylation (Ser79, AMP kinase phosphorylation site) (unpublished data). Although we cannot rule out possible involvement of AMP-kinase in the autoregulation of intrinsic glucose transport activity (18), our data suggest that glucose deprivation augments glucose uptake, which is mediated via an unidentified mechanism independent of GLUT4 translocation but dependent on PI 3-kinase activity (Fig. 9, A and B). Consistent with our findings, Abbud et al. (1) recently suggested the involvement of GLUT1 activation by AMP-kinase; however, the involvement of PI 3-kinase was not determined. Thus the nature of the intracellular signal that operates the glucose-dependent regulation of glucose uptake in C2C12 myotubes remains to be fully elucidated.

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