Glucose Transport in Human Skeletal Muscle Cells in Culture

Stimulation by Insulin and Metformin

Vivian Sarabia, Loretta Lam, Elena Burdett, Lawrence A. Leiter*, and Amira Klip
Division of Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8; and
**Department of Medicine, St. Michael's Hospital, Toronto, Ontario, Canada M5B 1W8*

Abstract

Primary human muscle cell cultures were established and the regulation of glucose transport was investigated. Primary cultures were allowed to proceed to the stage of myotubes through fusion of myoblasts or were used for clonal selection based on fusion potential. In clonally selected cultures, hexose (2-deoxyglucose) uptake into myotubes was linear within the time of study and inhibitable by cytochalasin B (IC_{50} = 400 nM). Cytochalasin B photolabeled a protein(s) of 45,000–50,000 D in a D-glucose–protectable manner, suggesting identity with the glucose transporters. In the myotube stage, the cells expressed both the GLUT1 and GLUT4 glucose transporter protein isoforms at an average molar ratio of 7:1. Preincubation in media of increasing glucose concentrations (range 5–25 mM) progressively decreased the rate of 2-deoxyglucose uptake. Insulin elevated 2-deoxyglucose uptake in a dose-dependent manner, with half maximal stimulation achieved at 3.5 nM. Insulin also stimulated the transport of the nonmetabolizable hexose 3-*O*-methylglucose, as well as the activity of glycogen synthase, responsible for nonoxidative glucose metabolism. The oral antihyperglycemic drug metformin stimulated the cytochalasin B-sensitive component of both 2-deoxyglucose and 3-*O*-methylglucose uptake. Maximal stimulation was observed at 8 h of exposure to 50 μM metformin, and this effect was not prevented by incubation with the protein-synthesis inhibitor cycloheximide. The relative effect of metformin was higher in cells incubated in 25 mM glucose than in 5 mM glucose, consistent with its selective action in hyperglycemic conditions in vivo. Metformin (50 μM for 24 h) was more effective than insulin (1 μM for 1 h) in stimulating hexose uptake and the hormone was effective on top of the stimulation caused by the biguanide, suggesting independent mechanisms of action. (*J. Clin. Invest.* 1992. 90:1386–1395.) Key words: cytochalasin β • glucose transport • insulin • metformin

Introduction

Skeletal muscle is the primary tissue responsible for glucose use in the postprandial state (1, 2). Glucose transport across the cell membrane is a rate-limiting step in glucose use by muscle (3, 4), and this step is exquisitely regulated by insulin and by the metabolic activity of this tissue, as well as by contractile activity (4). Glycogen synthesis, governed by the activity of the enzymes of the glycogen synthase complex, is also a key step in the regulation of nonoxidative glucose use (5). In animal models of diabetes, a decrease in muscle GLUT4,[1] glucose transporters accompanies the hyperglycemic state (7–9), and is thought to contribute to the insulin resistance detected both in vivo (during euglycemic clamps) (1) and in vitro (in isolated muscle preparations) (10). The nomenclature adopted for glucose transporters is based on their chronological discovery, according to G. Bell.

In human type II diabetes, insulin resistance is also observed (see reference 1), and it has been suggested that this resistance is related to functional defects in the muscle glucose transport system (11). Other studies have pointed to the glycogen synthase complex as an affected step in type II diabetes (12). For neither glucose transport nor glycogen synthesis are the precise defects known. In type II diabetes, a lack of change in the total muscle cell content of GLUT4 transporters has suggested that the defects may lie at the level of transporter activation or translocation to the cell membrane (13), but these defects remain to be detected. Difficulties in studying isolated muscles and in generating muscle subcellular fractions from human muscles are in large part responsible for the lack of further information.

Type II diabetes is commonly treated by a combination of diet and oral hypoglycemic agents. Of these, the sulfonylureas are more prevalently used in North America, but the biguanide, metformin, is highly used in Europe and increasingly in Canada. Sulfonylureas appear to act both by promoting insulin secretion and insulin action (14). In contrast, metformin appears primarily to improve peripheral glucose use (i.e., transport and/or use) (15, 16). The precise cellular basis of this action is unknown. Metformin has the therapeutic advantage over sulfonylureas that when used alone it poses no risk of hypoglycemia, since its effect is greater at higher than at normal glycemic values (see reference 17).

Isolated skeletal muscle preparations are unfortunately short lived for experimental manipulation, and they are invasive for human studies. In an attempt to define defects in glucose use, and to better understand the mechanism of action of metformin in human muscle, we have established human muscle cell cultures that are clonally selected for the myogenic fusion phenotype. We have previously characterized the kinetic parameters of 2-deoxyglucose uptake in these cells and compared them with the more widely studied rat L6 muscle cell line (18). The human muscle cells were shown to have a saturable glucose uptake system and to be more sensitive to insulin than the rat cell line. In the present communication we report that glucose transport in these clonally selected human muscle cell cultures is inversely regulated by the concentration of glucose in the medium, that insulin stimulates both glucose transport

Address correspondence to Dr. Amira Klip, Division of Cell Biology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.

Received for publication 25 September 1991 and in revised form 11 May 1992.

J. Clin. Invest.

0021-9738/92/10/1386/10 $2.00
Volume 90, October 1992, 1386–1395

1. For review see reference 6.

and glycogen synthase activity, and that metformin stimulates glucose transport by a mechanism independent of protein synthesis and of the presence of insulin. A preliminary report of this work has been presented (19).

Methods

Materials. β-D(+)-Glucose, porcine insulin, 2-deoxy-D-glucose, 3-*O*-methylglucose, gelatin, cytochalasin B, cytochalasin E, glycogen, and the sulfhydryl protease inhibitor E64 were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Deoxy-[^{3}H]glucose, 3-*O*-methyl-[^{3}H]glucose and ^{125}I-protein A were obtained from ICN Biomedicals (Costa Mesa, CA). [^{3}H]Cytochalasin B was from Amersham Corp. (Arlington Heights, IL). UDP-[^{14}C]glucose was obtained from New England Nuclear (Boston, MA). Metformin (*N,N*-dimethylbiguanide) was from Nordic Laboratories (Laval, Quebec, Canada). FBS, horse serum, αMEM, Ham's F-12 medium (F12),[1] containing 4.5 g/liter (25 mM) glucose, chick embryo extract (CEE), trypsin (0.25%), antibiotic antimycotic solution (10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 μg/ml amphotericin B) were all obtained from Gibco (Grand Island, NY). Collagenase (Worthington Type I) was obtained from Cooper Biomedical Diagnostic (Toronto, Ontario). BSA was from Boehringer Mannheim Corp. (Indianapolis, IN). Polyclonal antibodies to the GLUT1 and GLUT4 transporters were obtained from East-Acres Biologicals (Southbridge, MA) and are raised to COOH-terminal sequences specific to each transporter isoform. Tissue culture flasks, 60-mm dishes, 35-mm dishes, 24-well plates, and 96-well plates were all purchased from Nunc (Roskilde, Denmark). Cloning medium is a 1:1 mixture of αMEM:F12 media supplemented with 20% FBS, 1% CEE, and 1% antibiotic antimycotic solution. Growth medium is αMEM supplemented with 10% FBS and 1% antibiotic antimycotic solution. Fusion medium is αMEM with 2% FBS and 1% antibiotic antimycotic solution.

Primary human muscle cell cultures. Human skeletal muscle biopsies were obtained from the gracilus muscle of healthy nondiabetic adult patients during surgical procedures unrelated to this study. However, the procedure followed does not require surgical samples, as it is compatible with needle biopsy material. Clearance was obtained from the Hospital for Sick Children Human Experimentation Committee. Primary cultures were grown from satellite cells isolated from dissociated muscle tissue. The method of Yasin et al. (20) was adapted as described earlier (18, 21). Briefly, muscle samples (~ 0.5 g) were cleaned of excess fat and connective tissue in cold F12 medium and minced to a fine paste with a scalpel. Samples were then digested in a 60-mm dish for 15 min at 37°C with frequent agitation in a mixture of 0.15% trypsin, 0.1% collagenase, and 0.1% BSA. The supernatant containing single cells was decanted and FBS was added to a final concentration of 10% to stop enzymatic digestion. The remaining debris were subjected to another round of digestion (37°C for 15 min), the supernatants were pooled and cells were sedimented in a clinical centrifuge at 1,000 rpm for 10 min at 25°C. The cell pellet was resuspended in cloning medium and plated in 25-cm^2 flasks that were coated with 1% gelatin. A biopsy of 0.5 cm^3 can yield ~ 2×10^4 satellite cells. When studying primary cultures, a preplating step was introduced before growth in the 25-cm^2 flasks to diminish fibroblast content. Such primary cultures were allowed to fuse into myotubes and were used for determinations of glycogen synthase activity, [^{3}H]cytochalasin B photolabeling and content of GLUT1 and GLUT4 by Western blotting, since those assays require relatively large amounts of cells. For transport studies, primary cells were used for clonal selection as described next.

Clonal selection of muscle cells. When myoblasts of primary cultures reached 50–75% confluence (1–2 wk), cloning was initiated by an adaptation of the cloning procedure of Blau and Webster (22) as described previously (18, 21). Briefly, cells were trypsinized, counted, and diluted in cloning medium to 10 cells/ml, and then plated at a density of 1 cell/well in 96-well plates. When colonies were 50–75% confluent (2–4 wk, on average) they were transferred, 1 colony/well, to 24-well plates and allowed to proliferate. At 50–75% confluence (2–4 wk) single colonies were transferred to 60-mm culture dishes, grown to 75% confluence, frozen in 10% DMSO in cloning medium, and stored at −120°C. Small aliquots of all clones were grown to confluence in single wells of 24-well plates and exposed to fusion medium. Myogenic clones were selected by their ability to fuse into multinucleated myotubes under these conditions. On average, each 96-well plate will yield ~ 10 myogenic clones and the time required to obtain clones from biopsy material is ≥ 2 mo. For studies of glucose transport, cells from a fusing clone were seeded at a density of 3×10^4 cells/well in 24-well plates. All the experiments presented were performed on fused myotubes unless otherwise stated. Significant variability was observed in the absolute values of hexose transport when comparing different clones (see Results). This may originate from genetic diversity of the samples. However, all fusing cultures responded to insulin and to metformin as represented by the results herein. Where pertinent, results derived from several biopsies are illustrated.

2-Deoxyglucose and 3-O-methylglucose uptake. Effect of insulin: clonally selected human muscle cells were grown in 24-well plates, were allowed to fuse, and were preincubated for 5 h in αMEM containing 25 mM D-glucose in the absence of serum. The cells were then incubated with or without the desired concentration of insulin for 1 h at 37°C and then rinsed three times with hexose-free uptake solution consisting of the following (mM): 140 NaCl, 20 Hepes, 5 KCl, 2.5 $MgSO_4$, 1.0 $CaCl_2$; pH 7.4, 300 mosM and hexose uptake was determined in triplicate as previously described for L6 muscle cells using 2-deoxyglucose (23) or 3-*O*-methylglucose (24) as follows: cells were incubated at room temperature in uptake solution containing either 2-deoxy-[^{3}H]-glucose or 3-*O*-methyl-[^{3}H]glucose (10 μM) for 10 min or 30 s, respectively. Uptake was terminated by aspiration of the solution, immediately followed by rapid washing with 3 × 1 ml aliquots of ice-cold isotonic saline solution (containing 1.0 mM $HgCl_2$ in the case of 3-*O*-methylglucose uptake determinations). Cell-associated radioactivity was determined by cell lysis in 0.05 N NaOH, thorough scraping of cell lysates from plates, and scintillation counting of lysates in scintillation cocktail (Beckman HP; Beckman Instruments, Fullerton, CA). Non-carrier-mediated uptake was determined by parallel determinations in the presence of 5 μM cytochalasin B and was subtracted from all experimental values unless otherwise stated. Aliquots of cell lysates were saved for the determination of protein content by the method of Bradford (25) using the Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA). Transport is expressed throughout in pmol/min · mg protein.

For studies involving metformin, cells were incubated for 24 h (unless otherwise stated) in medium containing 25 mM glucose, 2% FBS, and the required concentration of metformin, before hexose uptake determination. In experiments where insulin was also used, the hormone was added during the last hour of incubation with metformin. Carrier-mediated hexose transport was then determined as above.

Glycogen synthase activity. The method of Guinovart et al. (26) was used as follows: primary cultures of human muscle cells (myotube stage) were incubated for 2 h in the absence of serum in medium containing 5 mM glucose, followed by a 1-h treatment with 1 μM insulin. The cultures were rinsed with cold saline solution, scraped with a rubber policeman, and collected by centrifugation. After resuspension in 1 ml of 250 mM glycylglycine, 10 mM EDTA, 125 mM KF, 1 mM DTT pH 7.0, the cells were disrupted by 60 strokes with a teflon pestle tissue homogenizer. The homogenate was centrifuged at 12,000 *g* for 10 min at 4°C and the supernatant was used for determination of protein and glycogen synthase activity using 200 μM UDP-[^{14}C]glucose in parallel incubations with 0.067 and 6.7 mM glucose-6-phosphate. The reaction was carried out for 15 min at 30°C, after which the amount of [^{14}C]-glucose incorporated into glycogen was determined as described by Thomas et al. (27) and modified by Guinovart et al. (26).

2. *Abbreviations used in this paper:* CEE, chicken embryo extract. F12, Ham's F-12 medium.

Crude membrane preparation, photolabeling with [^3H]cytochalasin B, and Western blotting. Crude membranes were prepared from confluent monolayers of fused human muscle cells, as follows: muscle cells from 10 flasks were rinsed with αMEM, then dislodged in 5 ml of αMEM using a rubber scraper. Cells were centrifuged for 10 min at 1,000 rpm in a clinical centrifuge followed by homogenization of the cell pellet in homogenization buffer (20 mM Hepes-Na pH 7.4, 250 mM sucrose, 2 mM EGTA, 5 mM NaN_3, 0.2 mM PMSF, 10 μM E64, 1 μM pepstatin A, 1 μM leupeptin) using a Dounce homogenizer (15–20 strokes). Total homogenates were centrifuged for 3 min at 6,000 rpm to remove nuclei and mitochondria. The supernatant was further centrifuged for 70 min at 200,000 *g* to yield the total membrane pellet. The protein yield was 80 μg protein/dish of fused myotubes.

Total cell membranes isolated as above were photolabeled with cytochalasin B, essentially as reported earlier (28, 29). Briefly, parallel samples of membranes were suspended at 3 mg protein/ml in 200 ml of "irradiation buffer" with the following composition (mM): (1 EDTA, 250 sucrose, 5 sodium phosphate, pH 7.4) containing 5 μM cytochalasin E, 1 μM of [^3H]cytochalasin B, and 0.39 M of either D- or L-glucose. The mixtures were irradiated with a 150-W Xenon lamp in a fluorescence spectrophotometer (650-40; Perkin Elmer Corp., Norwalk, CT) at 280 nm (15-nm slit) for four 15-min intervals separated by brief cooling on ice. After irradiation, membranes were sedimented by centrifugation at 178,000 *g* in a air-driven ultracentrifuge (Beckman Instruments) for 15 min and washed once with irradiation buffer by centrifugation. Labeled membrane pellets were then suspended in 60 ml of loading gel solution and immediately subjected to SDS-minigel electrophoresis on 10% polyacrylamide gels. After staining protein with Coomassie blue, gels were sliced into 2-mm segments, solubilized in 30% hydrogen peroxide at 75°C for 16 h, and counted in 12.5 ml of scintillation cocktail.

For Western blotting, total cell membranes were prepared from 5–10 flasks of primary muscle cell cultures, using the membrane isolation protocol described above. SDS-PAGE and Western blotting with αGLUT1 and αGLUT4 antibodies were carried out essentially as described earlier (30) but using the East-Acres antibodies (East-Acres Biologicals) defined above (see Fig. 2).

Statistics. These studies were carried out with cells from 11 different clones derived from two individuals. Within each experiment, each condition was assayed in quadruplicate. The results illustrated are of either mean±SE of (*n*) independent experiments or of one experiment representative of at least three independent ones. The latter format was chosen when there was significant variability in the absolute basal values in each experiment. In those instances, the results from all experiments are given in the text. Student's *t* test for paired data, or one-factor analysis of variance was applied where indicated.

Results

The glucose uptake system of human myotubes was first characterized by the uptake of 2-deoxyglucose and its interaction with the inhibitor cytochalasin B. Fig. 1 *A* shows that uptake of 2-deoxyglucose was linear for over 20 min. All determinations carried out in the present study were performed for either 5 or 10 min, in the linear range for all conditions tested. Uptake of 2-deoxyglucose involves both transport through the membrane hexose transporter and the subsequent phosphorylation of the sugar to 2-deoxyglucose-6-phosphate. At the concentrations used in this study (10 μM) the uptake of 2-deoxyglucose is limited by its transport across the cell membrane (18, 31). At higher concentrations (> 250 μM) phosphorylation becomes rate limiting (32). In Fig. 1 *A*, only the cytochalasin B–sensitive component of uptake is presented. The sensitivity of uptake to inhibition by this drug is illustrated in Fig. 1 *B*. In this experiment, the uptake assay was carried out in the presence of

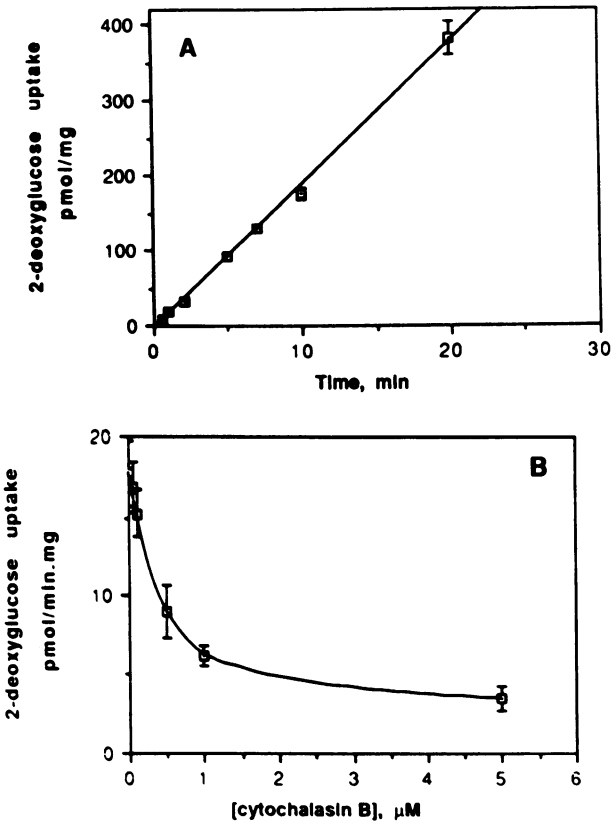

Figure 1. (*A*) Time course for the uptake of 2-deoxyglucose (10 μM) in fused human muscle cells. 2-Deoxyglucose uptake was determined for the indicated times as described in Methods. Results are of cytochalasin B–inhibitable uptake. Each point represents the mean of four parallel determinations. Data shown are of one experiment representative of three independent ones. (*B*) Dose–response curve of inhibition of 2-deoxyglucose uptake by cytochalasin B in fused human muscle cells. Uptake of 2-deoxyglucose was determined as described in Methods in the presence of the indicated concentrations of cytochalasin B. Each point represents the mean of four parallel determinations. Results are of one experiment representative of three independent ones.

the indicated concentrations of cytochalasin B. Half-maximal inhibition of 2-deoxyglucose uptake was obtained with 0.4 μM cytochalasin B. Maximal inhibition of transport was observed with 5 μM cytochalasin B (as 10 μM cytochalasin B caused no further inhibition). In contrast to cytochalasin B, its structural analogue cytochalasin E failed to inhibit transport at a concentration of 5 μM (not shown). These data are in close agreement with previous observations in rat L6 (31) and mouse C_2C_{12} (18) muscle cells and indicate that the large majority of hexose uptake occurs by facilitated diffusion through glucose transporters.

Cytochalasin B can photolabel both the GLUT1 and GLUT4 glucose transporters in muscle and fat cells (28, 33) by covalent incorporation upon irradiation with ultraviolet light. Photoactivation of the transporter proteins is responsible for this interaction (34). In experiments not shown, [^3H]-cytochalasin B reacted covalently with a component(s) corresponding to a molecular size of 50,000–45,000 D in cell extracts of human myotubes, and this reaction was largely prevented by D-glucose. These observations suggest that the glucose transporters of human skeletal muscle cells in culture

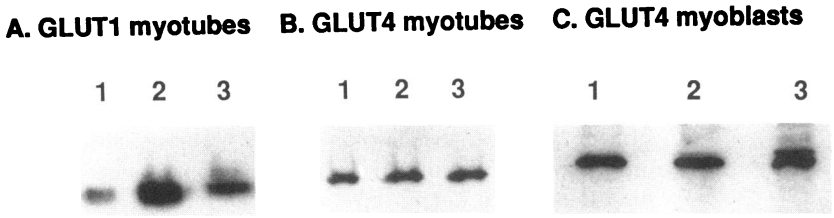

Figure 2. Western blot analysis of crude membranes of fused human myotubes (*A,B*) and unfused human myoblasts (*C*), reacted with α-GLUT1 (*A*) or α-GLUT4 (*B,C*) antibodies. The figure illustrates results of three different clones isolated from different muscle biopsies. Each lane was loaded with 160 μg protein of crude membranes. SDS-PAGE and Western blots were carried out essentially as described in reference 61. Exposure of filters to photographic films was carried out for 8 h in *A* and *B*, and 3.5 d in *C*.

belong to the same family as those expressed in adult skeletal muscle (36).

To investigate the specific isoforms of glucose transporters expressed in these cells, Western blot analysis was carried out on crude membrane preparations from cultures of fused myogenic primary cultures. αGLUT1 antibody reacted positively with a single component of 45,000 D in crude membranes from three independent cultures of fused human muscle cells (Fig. 2 *A*). This transporter isoform was also detected in rat skeletal muscle plasma membranes (35), in human muscle homogenates, and in human erythrocyte membranes (not shown). αGLUT4 antibody reacted similarly with a single component of 47,000 D in three independent cultures of fused human muscle cells (Fig. 2 *B*), as well as in rat and human muscle samples but not with in human erythrocytes, as expected (not shown). Although Fig. 2, *A* and *B* illustrate blots exposed to the film for the same time (8 h), the relative content of GLUT1/GLUT4 cannot be compared from the intensities of the bands since the respective antibodies can have different reactivities. Instead, the molar ratio of GLUT1/GLUT4 was estimated based on the following calculations: the densitometry of the bands of GLUT1 protein in the human muscle cell samples was compared with the densitometry of the human erythrocyte membranes run on the same gel and was converted to picomoles of transporter based on the determination of 400 pmol transporter/mg protein in the latter sample, measured by equilibrium binding of [^{3}H]cytochalasin B (36). The densitometry of the bands of GLUT4 of the human muscle samples was compared with the densitometry of the bands of rat muscle intracellular membranes and converted to picomoles of transporter based on the determination of 2.0 pmoles transporter/mg protein in the latter sample (also calculated by binding of [^{3}H]cytochalasin B). From these comparisons, GLUT1/GLUT4 molar ratios of 13:1, 3.4:1, and 5.6:1 were determined for crude (total) membranes of fused, primary muscle cell cultures derived from three independent muscle biopsies (absolute values of GLUT1 protein: 1.6, 0.3, and 0.7 pmol/mg total membrane protein; absolute values of GLUT4 protein: 0.12, 0.09, and 0.13 pmol/mg total membrane protein). By comparison, *crude membranes* from adult rat skeletal muscle were found to contain a GLUT1/GLUT4 molar ratio of 0.13:1 (absolute value of GLUT1 protein: 0.17 pmol/mg protein; absolute value of GLUT4 protein: 1.30 pmol/mg protein. For values in subcellular fraction see reference 37).[3]

Expression of the GLUT4 isoform is muscle- and fat-cell specific (see reference 6). This protein was detectable already at the myoblast stage in human muscle cells in culture, (Fig. 2 *C*) but this detection required 3.5 d of exposure to the film to achieve comparable levels of those observed in myotubes after 8 h of exposure. Hence, undifferentiated myoblasts appear to express lower levels of GLUT4 protein than differentiated myotubes. The calculated ratio of GLUT1/GLUT4 was roughly twofold higher in myoblasts than in myotubes within each clone, based on determinations of the picomoles of glucose transporters in samples of these cells, as described above.

The response of the glucose transport system to acute exposure to insulin was investigated next. Fig. 3 shows a typical insulin dose–response curve of 2-deoxyglucose uptake in human myotubes. A sigmoidal curve was obtained with maximal stimulation at 10^{-6} M insulin and half-maximal stimulation at 3.5×10^{-9} M insulin. When eight independent experiments were performed (each in quadruplicate) with clones derived from different individual biopsies, the stimulation caused by 10^{-7} M insulin was 35.5±3.4%. The mean basal transport rate was 18.4±2.4 pmol/min·mg and the mean insulin (10^{-7} M)-stimulated transport rate was 24.8±3.1 pmol/min·mg ($P < 0.001$, paired t test). To determine the effect of insulin directly on the transport step in human myotubes, uptake of the nonmetabolizable hexose 3-*O*-methylglucose was measured. Parallel experiments demonstrated that, at 10^{-7} M, insulin stimulated the cytochalasin B–sensitive component of 2-deoxyglucose uptake to the same extent as that of 3-*O*-methylglucose uptake (Table I). Nonspecific uptake (i.e., the cytochalasin B–insensitive component) was not affected by insulin. Thus, hexose *transport* through the glucose transporter is hormonally regulated.

In addition to glucose transport, glucose use in vivo is also regulated at the level of glucose incorporation into glycogen. This process is mediated by the glycogen synthase, which in human muscle is stimulated by insulin. It was therefore desirable to investigate whether human muscle cells in culture retain the ability to respond to the hormone with an increase in glycogen synthase activity. Table I shows that, indeed, after a 1-h incubation with the hormone, the activity of the enzyme assayed in a cell-free system was increased by 2.1-fold relative to the activity of the enzyme from untreated cells.

A characteristic of glucose uptake in adult skeletal muscle is its negative regulation by glycemia (38). Thus, in hyperglycemia, the glucose-clearance (i.e., transport) rate decreases, such that the net uptake of glucose into muscle may not change in

3. In skeletal muscle, the GLUT1 isoform is largely present in the perineurial cells (62), with ~ 40% of the immunoreactive transporter being detected in the plasma membrane of the myocyte proper (63). The potential presence of the GLUT3 isoform in these cells was not investigated due to the unavailability of antibodies to this protein at the time of performance of this study.

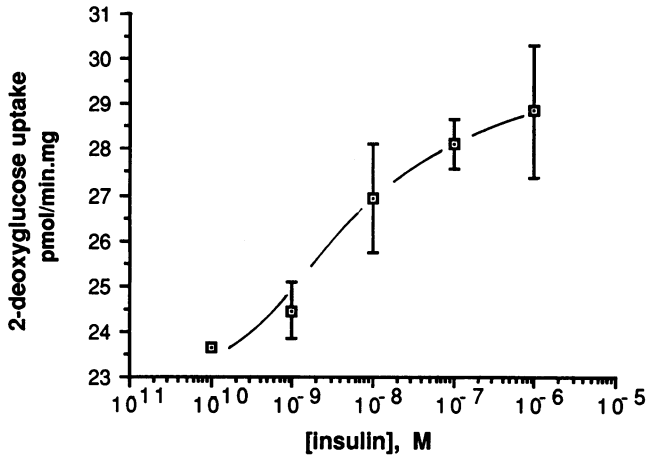

Figure 3. Dose–response curve of insulin action of 2-deoxyglucose uptake in human myotubes. Cells were depleted of serum in αMEM containing 25 mM glucose for 5 h and then incubated in the absence or presence of the indicated concentrations of insulin for 1 h at 37°C. Uptake of 2-deoxyglucose was determined as described in Methods. Results are of cytochalasin B–inhibitable uptake. Each point represents the mean of four parallel determinations within one experiment representative of three independent ones.

the face of a higher mass action of glucose (39). To demonstrate whether such a phenomenon (i.e., regulation of glucose transport activity by glucose itself) is a property of the muscle cells independent of other physiological influences, an in vitro model of hyperglycemia was created in human myotubes in culture. The cells were incubated with 5 (normoglycemic condition), 10, 15, or 25 mM glucose (hyperglycemic conditions) for 24 h at 37°C, after which the rate of 2-deoxyglucose transport was determined for 10 min in medium devoid of glucose. Fig. 4 shows the results of such experiments, expressed in relative units normalized to the value observed after preincubation in 5 mM extracellular glucose. Glucose uptake was reduced by 10, 25, and 45% in cells exposed for 24 h to medium containing 10, 15, and 25 mM glucose, respectively. The results of three independent experiments showed an average reduction of 41% in 25 relative to 5 mM glucose (the mean uptake in 5 mM glucose was 36.7±4.7 compared with 21.8±1.0 pmol/min · mg protein in 25 mM glucose). Hence, glucose uptake is downregulated by hyperglycemia in human skeletal muscle cells in vitro. This is consistent with the response of muscle to hyperglycemia observed in vivo in diabetic individuals.

Type II diabetes is often treated with oral hypoglycemic agents, of which metformin is increasingly being used in North America. The blood glucose–lowering effect of metformin occurs without changes in insulin secretion, suggesting that the drug may enhance glucose use by the muscle (the primary consumer of glucose in the postprandial state). This possibility was directly assessed by investigating the effect of metformin on hexose uptake in human myotubes in culture. Unless otherwise stated, the response to the drug was measured in cells preincubated with 25 mM glucose to mimic observed glycemic values of diabetic individuals. Fig. 5 illustrates the averaged results of five independent experiments, indicating that exposure to metformin (50 μM) for 24 h elevated the rate of 2-deoxyglucose uptake, specifically of the cytochalasin B–inhibitable component. Thus, metformin stimulated carrier-mediated hexose uptake rather than increasing nonspecifically the membrane permeability to glucose. When five independent experiments were examined, each performed at least in triplicate, the stimulation caused by 50 μM metformin was 33.8±6.1% ($P < 0.01$, paired t test). This value is in agreement with the stimulation of whole body glucose use caused by metformin in vivo (17). To establish whether the stimulation by metformin of hexose uptake was due to increased transport of hexose through the glucose transporter or to the subsequent phosphorylation of the sugar, the effect of metformin on the uptake of 3-*O*-methylglucose was determined. The ratio of metformin stimulated to basal uptake was the same for 3-*O*-methylglucose as for 2-deoxyglucose (Table I). This demonstrates that metformin stimulates transport through the glucose transporter and that this effect is quantitatively reflected in the stimulation of uptake of 2-deoxyglucose.

Fig. 6 shows the results of three independent experiments analyzing the metformin concentration–dependent stimulation of 2-deoxyglucose uptake, expressed as percentage stimulation above control values. In humans, therapeutic circulating levels of metformin reach values ranging from 12 to 100 μM (40). At 50 μM metformin in vitro, 2-deoxyglucose uptake was stimulated by 25–30%. Maximal stimulation of uptake (135%) was achieved with 800 μM of the drug. At any concentration tested, metformin did not alter the amount of protein in the cultures after a 24-h incubation period.

The time course of metformin action is shown in Fig. 7.

Table I. Stimulation by Insulin of Hexose Uptake and Glycogen Synthase Activity

Preincubation	2-Deoxyglucose uptake	3-*O*-Methylglucose transport	Glycogen synthase activity	
	relative U	*relative U*	*L/H*	*relative U*
Control	1.0	1.0	0.11±0.04 (3)	1.0
Insulin	1.36±0.03 (8)	1.35±0.17 (3)	0.23±0.09 (3)	2.1
Metformin	1.34±0.06 (5)	1.33±0.06 (3)	ND	

Preincubation conditions: insulin concentration and time: 0.1 μM for 1 h for 2-deoxyglucose or 3-*O*-methylglucose uptake; 1 μM for 1 h for glycogen synthase. Other details as outlined in the Methods. Metformin concentration and time: 50 μM for 24 h. These preincubations were followed by assays of 2-deoxyglucose uptake (10 min), 3-*O*-methylglucose transport (30 s), or glycogen synthase activity. L/H refers to the ratio of glycogen synthase activity in low (0.067 mM) and high (6.7 mM) glucose-6-phosphate. ND, not determined. Results are of (*n*) independent experiments, each performed in quadruplicate for transport determinations and in triplicate for glycogen synthase determinations.

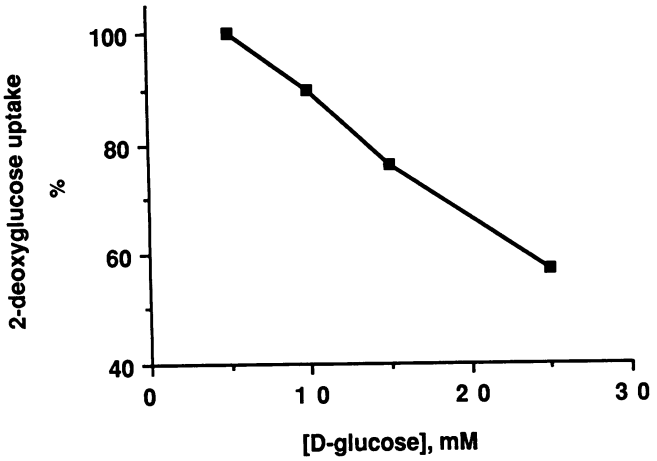

Figure 4. Effect of glucose preincubation on 2-deoxyglucose transport. Human myotubes were incubated in αMEM containing 2% FBS with the indicated concentrations of glucose for 24 h, followed by measurement of 2-deoxyglucose uptake in glucose-free medium as described in Methods. Results are of cytochalasin B–inhibitable uptake and are expressed as percent of the uptake in cells incubated in 5 mM glucose. Results are of four replicate determinations within a single experiment. Three similar independent experiments were carried out with 5, 15 mM, and 25 mM glucose (see text).

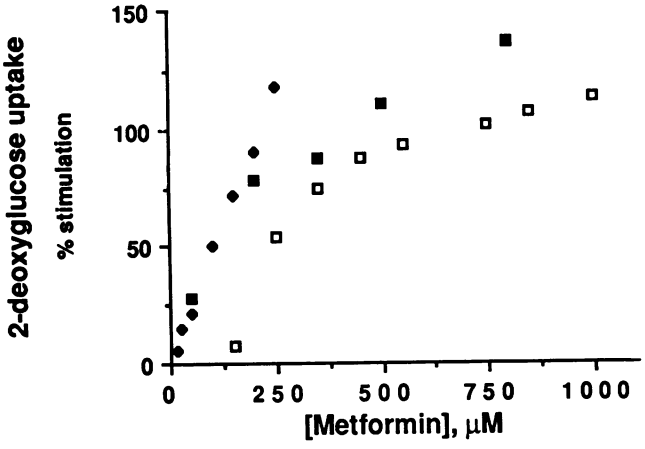

Figure 6. Concentration dependence curve for metformin-stimulated 2-deoxyglucose transport. Human myotubes were incubated for 24 h with the indicated concentrations of metformin in αMEM containing 2% FBS and 25 mM glucose. Uptake of 2-deoxyglucose was determined as described in Methods. Results are of cytochalasin B–inhibitable uptake and expressed as percent stimulation above control (i.e., not treated with metformin). The results of three independent experiments are illustrated, each performed in triplicate. □, Experiment 1; ■, experiment 2; ♦, experiment 3.

Metformin (50 μM) stimulated transport within 4 h and maximal stimulation was achieved by 8 h. To determine the possible role of de novo protein synthesis in this response, human myotubes were incubated for 24 h with metformin and the protein synthesis inhibitor cycloheximide (2 μg/ml). This concentration of cycloheximide and time of exposure were previously shown to inhibit the stimulation of glucose transport caused by chronic 24-h exposure to insulin in L6 muscle cells (41). In the presence of cycloheximide, 50 μM metformin was able to stimulate 2-deoxyglucose uptake by 28±2.5% in three independent experiments, comparable to the stimulation in the absence of the inhibitor (34±6%, n = 5), indicating that the biguanide stimulates glucose uptake by a mechanism that is independent of protein synthesis.

The above results were obtained in medium containing 15 mM glucose. We next compared the effectiveness of metformin in low and high glucose–containing media. Fig. 8 presents the averaged results of three independent experiments investigating the response to the drug in normoglycemic (5 mM) and hyperglycemic (25 mM) conditions. The biguanide stimulated 2-deoxyglucose uptake above control levels significantly only

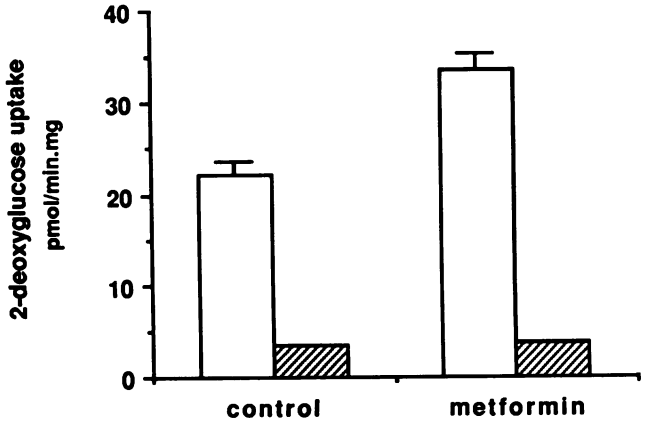

Figure 5. Effect of metformin on cytochalasin B–inhibitable 2-deoxyglucose uptake. Human myotubes were incubated for 24 h in αMEM containing 2% FBS and 25 mM glucose in the absence or presence of 50 μM metformin. 2-Deoxyglucose uptake was determined as described in Methods in the absence (*open bars*) or presence (*cross-hatched bars*) of 5 μM cytochalasin B (CB). Results are the mean±SE of five independent experiments, each performed in triplicate.

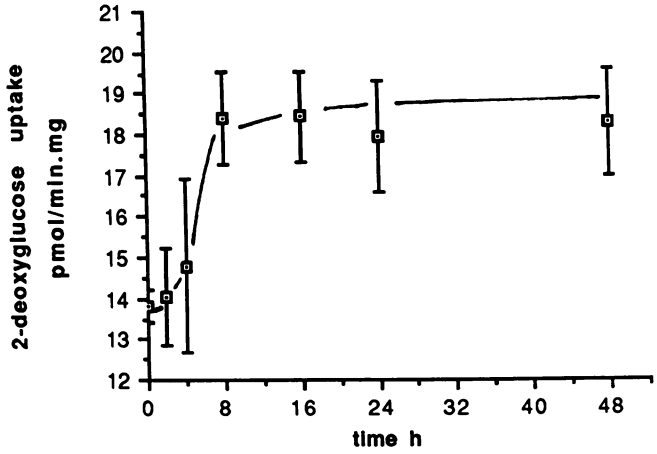

Figure 7. Time course of metformin stimulation of 2-deoxyglucose transport. Human myotubes were incubated with 50 μM metformin for the indicated periods of time in αMEM containing 2% FBS and 25 mM glucose. Uptake of 2-deoxyglucose was determined as described in Methods. Results are of cytochalasin B–inhibitable uptake, showing the mean of quadruplicate determinations within one experiment representative of two.

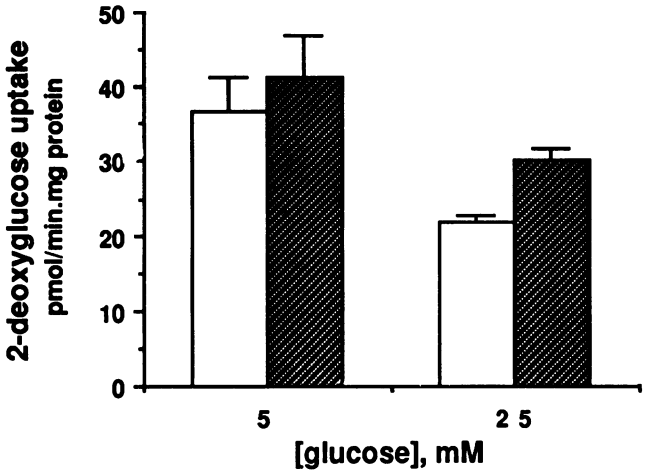

Figure 8. Effect of metformin on 2-deoxyglucose transport in normal and high glucose media. Human myotubes were incubated without (*open bars*) or with 50 μM metformin (*cross-hatched bars*) for 24 h in αMEM containing 2% FBS and either 5 or 25 mM glucose. Uptake of 2-deoxyglucose was determined as described in Methods. Results are of cytochalasin B–inhibitable uptake, expressed in absolute transport units (pmol/min · mg protein). Results are the mean±SE of three independent experiments, each carried out in triplicate. □, Control; ▨, metformin.

in 25 mM glucose media (net gains of 4.6±0.9 and 8.2±2.2 pmol/min · mg protein, $n = 3$, in normoglycemic and hyperglycemic media, respectively). Moreover, since the basal rate of transport was significantly diminished by high glucose incubation, metformin produced a greater relative increase in hyperglycemic medium (38.3±10.6%) than in normoglycemic medium (11.5±0.9%). This suggests that the "responsiveness" to metformin is higher in hyperglycemic than in normoglycemic medium conditions.

The combined effects of maximal concentrations of metformin and insulin were also tested. Human myotubes were incubated in αMEM containing 2% FBS and 25 mM glucose with 1 μM insulin for 1 h, 800 μM metformin for 24 h, or 800 μM metformin for 24 h followed by 1 h with metformin and 1 μM insulin. 2-Deoxyglucose uptake was then determined for 5 min. The results of three independent experiments, with each point performed in quadruplicate, indicated that these maximal concentrations of insulin on top of metformin treatment caused a further stimulation of 2-deoxyglucose uptake: basal transport was 7.2, 7.4, and 15.8 pmol/min · mg protein in each experiment; transport in the presence of 800 μM metformin was 15.7, 15.6, and 22.1 pmol/min · mg; transport in the presence of 1 μM insulin was 8.4, 8.5, and 16.8 pmol/min · mg protein, and transport in the presence of 24 h metformin followed by 1 h insulin + metformin was 17.9, 17.3, and 25.2 pmol/min · mg protein, respectively. Thus, in spite of the variability among experiments, within each one insulin caused a further stimulation over the effect of metformin. One-factor analysis of variance indicated that the effect of insulin alone versus metformin plus insulin was significantly different at 95% (Dunnett $t = 2.429$). By this test the difference between the effects of metformin versus metformin plus insulin did not reach the 95% confidence interval. However, paired Student's t test analysis of the metformin alone versus metformin plus insulin groups gave a $P < 0.05$. These findings therefore are suggestive, but do not prove, that metformin and insulin may act through independent pathways. It is also interesting to note that the stimulatory effect of 50 μM metformin (a therapeutically relevant concentration) after 24 h was similar to that of acute insulin action; however, the effect of 800 μM metformin (a pharmacological dose) largely surpassed the acute response to the hormone.

Discussion

Skeletal muscle is the primary tissue disposing of glucose in the presence of insulin and the response to the hormone is diminished in type II diabetic individuals. The molecular basis for the defect is unknown and its study would require investigations in human muscle preparations. In contrast to studies with animal tissue, isolated whole muscles from humans clearly cannot be studied. Isolated muscle strips from humans have recently been implemented to measure 3-*O*-methylglucose uptake and were shown to respond to maximal insulin concentrations in vitro with a twofold stimulation (42). However, the isolation of such strips is possible only during surgical procedures, which limits their availability. Furthermore, the isolation requires intact excision to preserve membrane integrity and the preparation has a relatively short survival in vitro. A further complication of studies with excised muscle is the heterogeneous fiber composition of each muscle. Hexose uptake and its stimulation by insulin or exercise have been shown to vary in different muscle types depending on their contractile twitch and aerobic metabolism (see reference 4). Hence, there is a genuine need to establish a system of human muscle cells that is viable for prolonged time periods, has genetic constancy, and can be prepared from muscle biopsy material.

Cells in culture have proven to be appropriate for glucose transport studies (43) since they grow in monolayers, allowing for even exposure to substrate and for determinations of initial rates of uptake. Because the cells are clonally selected, they represent a homogeneous population. Several muscle cell lines of animal origin are currently available (rat L6 and mouse C_2C_{12} of skeletal muscle origin and mouse BC3H1 of brain tumour origin). Glucose uptake has been previously characterized in these cells and was found to be saturable, carrier-mediated, and regulated by insulin (18, 44, 45). There are no muscle cell lines of human origin that have been equally characterized. Although the terminally differentiated myotubes of mature muscle have lost the potential to divide, techniques have emerged in recent years to culture muscle cells from adult tissue biopsies, based on the myogenic potential of satellite cells (20, 22). In the present study, we established human muscle cell cultures from satellite cells of adult muscle and investigated the properties of their glucose transport system as well as its regulation by insulin and the antihyperglycemic drug metformin.

Metformin is used in the treatment of type II diabetes. The mechanism of action of this biguanide is unknown, but much indirect evidence suggests that its major effect is on glucose use. Several studies with humans and animals under euglycemic–hyperinsulinemic clamp have demonstrated that metformin increases peripheral glucose uptake (15–17, 46, 47), although the results in humans has been disputed in one study (48). On

average the increase is 27%. Studies on rat diaphragm muscles isolated from diabetic animals showed that in vitro treatment with metformin increases insulin-dependent glucose uptake (49). In fragments of nondiabetic human skeletal muscle, exposure to metformin increased by ~ 30% the insulin-dependent glucose disappearance from the medium when the hexokinase was first depressed by sodium butyrate (50). The only study investigating the effect of metformin on hexose *transport* into muscle measured the uptake of 3-*O*-methylglucose in soleus muscle of streptozotocin-treated mice after in vivo administration of metformin (51). Transport was increased by 20% at both submaximal and maximally stimulating insulin concentrations.

In the present study, the characteristics of the glucose transport system of human muscle cells in culture were investigated at the stage of fused myotubes. In a recent study, Mesmer and Lo (52) investigated the kinetic parameters of hexose uptake in human myoblasts before cell confluence. We chose to study fused myotubes clonally selected for fusion potential to achieve a closer comparison with adult skeletal muscle and to avoid complications due to regulation of glucose transport by growth and cell cycle stage. Hexose transport in these myotubes was inhibited by submicromolar concentrations of cytochalasin B, indicating that uptake occurs through a transporter of the facilitated diffusion family of transporters (53). This ligand, when used as a D-glucose protectable covalent label, identified the glucose transporters as a polypeptide(s) of 45,000–50,000 D, exactly in the range previously determined for rat skeletal muscle (28). Insulin stimulated the uptake of both 2-deoxyglucose and 3-*O*-methylglucose to the same extent, suggesting an effect at the level of hexose transport. Although the stimulation was small, it must be recognized that the increase in hexose transport by insulin in isolated human muscle strips is only ~ 150% above the basal value (42). Thus, isolated muscle systems appear to have a lower responsiveness than the muscle in vivo. The explanation for the even lower responsiveness of human muscle cell cultures is not known at present, but it may be related to the lower ratio of GLUT4/GLUT1 transporters expressed in the cultures compared with the mature muscle fibers. In addition, it is possible that the lack of innervation and the growth conditions in culture prevent the development of the full response to the hormone.

The stimulation by insulin of the uptake of 2-deoxyglucose was equivalent to that of 3-*O*-methylglucose, suggesting that under the conditions of this study (10 μM hexose, 10-min uptake) 2-deoxyglucose transport is rate limiting over its subsequent phosphorylation. The hormone also increased, in addition, the activity of the glycogen synthase, suggesting that these key regulatory actions of insulin are preserved in the muscle cell cultures (Table I).

Exposure of fused muscle cells to metformin caused an increase only in the cytochalasin B–sensitive portion of 2-deoxyglucose uptake (Fig. 5), indicating that the drug acts by raising glucose uptake through the glucose transport system. In addition, metformin augmented the uptake of the nonmetabolizable sugar 3-*O*-methylglucose (Table I) to approximately the same extent as the uptake of 2-deoxyglucose, suggesting a stimulation of hexose transport. This stimulation was both concentration and time dependent (Figs. 6 and 7, respectively). Increased hexose uptake was obtained at concentrations of metformin (50–100 μM) in the range of circulating levels of metformin found in the blood of patients (12–100 μM) after the standard oral dose of 1.5 g/d (40). Metformin is not metabolized in humans so other derivatives are ruled out as mediators of its biological effects. Most studies reported here were carried out using 50 μM metformin, a concentration in the therapeutic range. It is noteworthy that 50 μM metformin was as effective and 800 μM metformin was even more effective than maximal concentrations of insulin in stimulating glucose uptake. This suggests that the drug may act through a mechanism that is different from that of insulin (see below) or that the same mechanism is not fully used by insulin. This was further confirmed by the additive effects of maximal concentrations of insulin and metformin. A plausible explanation for the larger effect of metformin than of insulin could be related to the high GLUT1/GLUT4 ratio observed in these cells, if insulin were to act primarily through GLUT4 transporters and metformin through GLUT1 transporters in these cells (as is the case in rat L6 myotubes). This conclusion is yet to be experimentally supported for human muscle cells in culture.

The time course of metformin stimulation of hexose transport was relatively slow with maximal stimulation occuring by 8 h and half-maximal stimulation at 4–5 h (Fig. 7). This is comparable to the stimulation of glucose uptake by glucose deprivation in rat L6 muscle cells (24), which requires hours to develop. In contrast, the acute effects of insulin occur within minutes in both L6 and human muscle cells in culture. The response to metformin in human muscle cells in culture did not require ongoing protein synthesis, reminiscent of the response to glucose deprivation (41). The latter response is largely mediated by an increase in glucose transporter number at the cell membrane (41). The intracellular signaling by metformin appears to be distinct from that by insulin, based on the different lag periods preceding the respective responses. Alternatively, metformin may require time to reach an intracellular site at sufficient concentrations. This possibility remains to be determined. The drug is a weak base and it is expected that it may penetrate slowly the cell lipid bilayer. Lack of available radioactively labeled metformin precludes at present the determination of the site of binding or action of metformin. Even though insulin has a secondary (late) stimulation of glucose transport, the action of metformin also differs from this late hormonal response, which is dependent on de novo protein synthesis (41).

The molecular mechanism(s) responsible for the stimulation of hexose transport by either insulin or metformin in human muscle cells remains to be determined. Effects of metformin on the number of insulin receptors in other cells have been controversial (see reference 17) and have been found not to correlate with metformin action (54). The lack of a requirement of protein synthesis for metformin action in human muscle cells is suggestive of either activation or translocation of glucose transporters to the plasma membrane. Unfortunately, the slow rate of growth of primary cultures relative to cell lines dictates that small amounts of material are obtained, which preclude carrying out detailed biochemical analysis needed to assess the subcellular distribution of glucose transporters. That approach has been used with the more rapidly growing rat L6 muscle cells, in which we have shown that insulin induces translocation of glucose transporters (cytochalasin B–binding sites) (55) akin to that observed in rat skeletal muscle (56). Recently we reported that metformin treatment also increases

glucose uptake and the amount of GLUT1 transporters in isolated plasma membranes from rat L6 muscle cells (57), without changing the net content of either GLUT1 or GLUT4 proteins (58). Human muscle cells in culture (used in this study) as well as rat L6 muscle cell lines express higher ratios of GLUT1/GLUT4 transporters than do adult rat or human muscles (58). Other insulin-responsive muscle cell lines (BC_3H1, C_2C_{12}, and G_8) have been reported to express detectable levels of only GLUT1 (59). Hence, it appears that human primary muscle cell cultures and rat L6 muscle cells are the only muscle cell culture systems known to express the muscle-specific GLUT4 glucose transporter, although a direct comparison of all listed cell types under the same experimental conditions would be required to fully ascertain this point. In preliminary experiments we were unable to detect GLUT4 protein in isolated plasma membranes and microsomes from a clone of differentiated mouse C_2C_{12} myotubes (Mitsumoto, Y., and A. Klip, unpublished observations).

The qualitative differences between cultured human muscle cells and adult human skeletal muscle may be related to the less mature state of the cells in culture, to the lack of innervation in culture, or to other conditions typical of the culture procedure. (Indeed, GLUT1 and GLUT4 transporters are under developmental control, the ratio GLUT4/GLUT1 increasing notably after cell fusion as seen here both in human muscle cells in culture and in rat L6 muscle cells [60]). These alternatives are being investigated. Nonetheless, the cells in culture retain the ability to respond to insulin and, as shown here, they respond to metformin with an increase in glucose uptake. When this manuscript was being prepared, Matthaei et al. (61) reported that in isolated rat adipocytes metformin potentiated the insulin-induced recruitment of glucose transporters. In the short time of exposure to the drug studied (2 h), metformin did not cause stimulation of glucose transport in those cells. These results are not incompatible with our observations in human muscle cells. Indeed, exposures longer than 2 h were required to detect the stimulation of hexose transport by metformin in the latter cells and this effect was additive to that of insulin. Since metformin therapy is associated with continuous treatment that maintains elevated levels of the drug throughout the day and night, it is likely that glucose transport is stimulated in vivo continuously by the biguanide and that the drug further potentiates the action of insulin in the postprandial state.

In conclusion, human muscle cells in culture of clonal origin and differentiated to the state of myotubes are a viable model of human cells that retain the ability to respond to insulin and glucose. In contrast to currently studied muscle cell lines (which are of animal origin), these clonally selected human cells are of primary origin and are not chemically transformed. As a consequence, they are likely to represent more faithfully events occurring in human muscle. Even though the cells are slow growing and yield limited material for biochemical studies compared with established cell lines of animal origin, they may be a useful model to study genetic defects in glucose transport and metabolism. These cells express the GLUT4 glucose transporter and respond to insulin with a stimulation of hexose transport and glycogen synthase activity. In these cells, the hitherto unknown mechanism of action of the oral hypoglycemic drug metformin is shown to involve stimulation of glucose transport through the glucose transport system. It must be considered possible, however, that in vivo metformin may also act at other sites in addition to muscle (i.e., fat, liver) such that a combination of its actions will determine its final effect on circulating blood glucose levels.

Acknowledgments

We thank Toolsie Ramlal, B.Sc., Ulla-Maija Koivisto, M.Sc., Henry Klamut, Ph.D., and Lawrence Mandarino, Ph.D., for valuable advice in these studies.

This work was supported by a University-Industry grant from the Medical Research Council of Canada and Nordic Laboratories, Canada. A. Klip was the recipient of a Medical Research Council Scientist Award.

References

1. DeFronzo, R. 1988. The triumvirate: β-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes.* 37:667–686.

2. Baron, A. D., G. Brechtel, P. Wallace, and S. V. Edelman. 1988. Rates and tissue sites of non-insulin-and insulin-mediated glucose uptake in humans. *Am. J. Physiol.* 255:E769–E774.

3. Elbrink, J. I., and I. Bihler. 1975. Membrane transport: its relation to cellular metabolic rates. *Science (Wash. DC).* 188:1177–1183.

4. Klip, A., and M. R. Paquet. 1990. Glucose transport and glucose transporters in muscle and their metabolic regulation. *Diabetes Care.* 13:228–243.

5. Mandarino, L. J., K. S. Wright, L. S. Verity, J. Nichols, J. M. Bell, O. G. Kolterman, and H. Beck-Nielsen. 1987. Effects of insulin on human skeletal muscle pyruvate dehydrogenase, phosphofructokinase, and glycogen synthase. *J. Clin. Invest.* 80:655–663.

6. Gould, G. W., and G. I. Bell. 1990. Facilitative glucose transporters: an expanding family. *Trends Biochem. Sci.* 15:18–23.

7. Garvey, T. W., T. P. Huecksteadt, and M. J. Birnbaum. 1989. Pretranslational suppression of an insulin-responsive glucose transporter in rats with diabetes mellitus. *Science (Wash. DC).* 245:60–63.

8. Bourey, R. E., L. Koranyi, D. E. James, M. Mueckler, and M. A. Permutt. 1990. Effects of altered glucose homeostasis on glucose transporter expression in skeletal muscle of the rat. *J. Clin. Invest.* 86:542–547.

9. Klip, A., T. Ramlal, G. Cartee, E. Gulve, and J. O. Holloszy. 1990. Insulin-induced recruitment of glucose transporters to the plasma membrane in skeletal muscle from diabetic rats. *Biochem. Biophys. Res. Commun.* 172:728–736.

10. Wallberg-Henriksson, H., and J. O. Holloszy. 1985. Activation of glucose transport in diabetic muscle: responses to contraction and insulin. *Am. J. Physiol.* 249:C233–C237.

11. Ciaraldi, T. P., O. G. Kolterman, J. A. Scarlet, M. Kao, and J. M. Olefsky. 1982. Role of glucose transport in the postreceptor defect of non-insulin-dependent diabetes mellitus. *Diabetes.* 31:1016–1022.

12. Thorburn, A. W., B. Gumbiner, F. Bulacan, G. Brechtel, and R. R. Henry. 1987. Multiple defects in muscle glycogen synthesis in non-insulin dependent diabetes mellitus. *J. Clin. Invest.* 87:489–495.

13. Pedersen, O., J. F. Bak, P. H. Andersen, S. Lund, D. E. Moller, J. S. Flier, and B. B. Kahn. 1990. Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes.* 39:865–870.

14. Lebovitz, H. E., and R. Pasmantier. 1990. Combination insulin-sulfonylurea therapy. *Diabetes Care.* 13:667–675.

15. Hother-Nielsen, O., O. Schmitz, P. H. Andersen, H. Beck-Nielsen, and O. Pedersen. 1989. Metformin improves peripheral but not hepatic insulin action in obese patients with type II diabetes. *Acta. Endocrinol.* 120:257–265.

16. Prager, R., G. Schernthaner, and H. Graf. 1986. Effect of metformin on peripheral insulin sensitivity in non-insulin dependent diabetes mellitus. *Diabete Metab.* 12:346–350.

17. Klip, A., and L. A. Leiter. 1990. Cellular mechanism of action of Metformin. (Invited review.) *Diabetes Care.* 13:696–704.

18. Sarabia, V., T. Ramlal, and A. Klip. 1990. Glucose uptake in human and animal muscle cells in culture. *Biochem. Cell Biol.* 68:536–542.

19. Klip, A., V. Sarabia, T. Ramlal, P. J. Bilan, and L. A. Leiter. 1990. Glucose transport in human muscle cells in culture: stimulation by insulin and metformin. *Diabetes.* 39(Suppl. 328):82A.

20. Yasin, R., G. Van Beers, K. C. E. Nurse, S. Al-Ani, D. N. Landon, and E. J. Thompson. 1977. A quantitative technique for growing human adult skeletal muscle in culture starting from mononucleated cells. *J. Neurol. Sci.* 32:347–360.

21. Sarabia, V., and A. Klip. 1989. Regulation of cytosolic Ca^{2+} in clonal human muscle cell cultures. *Biochem. Biophys. Res. Commun.* 165:1130–1137.

22. Blau, H. M., and C. Webster. 1981. Isolation and characterization of human muscle cells. *Proc. Natl. Acad. Sci. USA.* 78:5623–5627.

23. Klip, A., and T. Ramlal. 1987. Protein kinase C is not required for insulin stimulation of hexose uptake in muscle cells in culture. *Biochem. J.* 242:131–136.

24. Walker, P. S., T. Ramlal, J. A. Donovan, T. P. Doering, A. Sandra, A. Klip, and J. E. Pessin. 1989. Insulin and glucose-dependent regulation of the glucose transport system in the rat L6 skeletal muscle cell line. *J. Biol. Chem.* 264:6587–6595.

25. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. *Anal. Biochem.* 72:248–254.

26. Guinovart, J. J., A. Salavert, J. Massage, C. J. Ciudad, E. Salsas, and E. Iltarte. 1979. Glycogen synthase: a new activity ratio assay expressing a high sensitivity to the phosphorylation state. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 106:284–288.

27. Thomas, J. A., K. K. Schlender, and J. Larner. 1968. A rapid filter paper assay for UDP-glucose-glycogen glucosyltransferase including an improved biosynthesis of UDP-[^{14}C]glucose. *Anal. Biochem.* 25:486–499.

28. Klip, A., D. Walker, K. J. Ransome, D. W. Schroer, and G. E. Lienhard. 1983. Identification of the glucose transporter in rat skeletal muscle. *Arch. Biochem. Biophys.* 226:198–205.

29. Klip, A., M. Deziel, and D. Walker. 1984. Chemical identity of the glucose transporter with the [^{3}H]cytochalasin B-photolabelled component of human erythrocyte membranes. Equal sensitivity to trypsin and endoglycosidase F. *Biochem. Biophys. Res. Commun.* 122:218–224.

30. Koivisto, U. M., H. Martinez-Valdez, P. J. Bilan, E. Burdett, T. Ramlal, and A. Klip. 1991. Differential regulation of the GLUT-1 and GLUT-4 glucose transport systems by glucose and insulin in L6 muscle cells in culture. *J. Biol. Chem.* 266:1615–1621.

31. Klip, A., W. J. Logan, and G. Li. 1982. Hexose transport in L6 muscle cells. Kinetic properties and the number of [^{3}H]cytochalasin B binding sites. *Biochim. Biophys. Acta.* 687:265–280.

32. Jacobs, A. E. M., A. Ossterhof, and J. H. Veerkamp. 1990. 2-deoxy-D-glucose uptake in cultured human muscle cells. *Biochim. Biophys. Acta.* 1051:230–236.

33. James, D. E., R. Brown, J. Navarro, and P. F. Pilch. 1988. Insulin-regulatable tissues express a unique insulin-sensitive glucose transporter protein. *Nature (Lond.).* 333:183–185.

34. Deziel, M., W. Pegg, E. Mack, A. Rothstein, and A. Klip. 1984. Labelling of the human erythrocyte glucose transporters with [^{3}H]cytochalasin B occurs via protein photoactivation. *Biochim. Biophys. Acta.* 772:403–406.

35. Douen, A. G., T. Ramlal, S. Rastogi, P. J. Bilan, G. Cartee, M. Vranic, J. O. Holloszy, and A. Klip. 1990. Exercise induces recruitment of the 'insulin responsive glucose transporter'. Evidence for distinct intracellular insulin-and-exercise-recruitable transporter pools in skeletal muscle. *J. Biol. Chem.* 265:13427–13430.

36. Bilan, P. J., and A. Klip. 1990. In vitro glycation of the human erythrocyte glucose transporter and its functional consequences. *Biochem. J.* 268:661–667.

37. Marette, A., H. S. Hundal, and A. Klip. 1991. Regulation of glucose transporter proteins in skeletal muscle. *Proc. Symp. Diabetes Mellitus and Exercise.* In press.

38. Klip, A., A. Marette, D. Dimitrakoudis, T. Ramlal, A. Giacca, Z. Q. Shi, and M. Vranic. 1992. Effect of diabetes on glucoregulation: from glucose transporters to glucose metabolism in vivo. *Diabetes Care.* In press.

39. Kelley, D. E., and L. J. Mandarino. 1990. Hyperglycemia normalizes insulin-stimulated skeletal muscle glucose oxidation and storage in noninsulin-dependent diabetes mellitus. *J. Clin. Invest.* 86:1999–2007.

40. Pentikainen, P. J., P. J. Neuvonen, and A. Penttila. 1979. Pharmacokinetics of metformin after intravenous and oral administration to man. *Eur. J. Clin. Pharmacol.* 16:195–202.

41. Walker, P. S., T. Ramlal, V. Sarabia, U. M. Koivisto, P. J. Bilan, J. E. Pessin, and A. Klip. 1990. Glucose transport activity in L6 muscle cells is regulated by the coordinate control of subcellular glucose transporter distribution, biosynthesis, and mRNA transcription. *J. Biol. Chem.* 265:1516–1523.

42. Dohm, G. L., E. B. Tapscott, W. J. Pories, D. J. Dabbs, E. G. Flickinger, D. Meelheim, T. Fushiki, S. M. Atkinson, C. W. Elton, and J. F. Caro. 1988. An in vitro human muscle preparation suitable for metabolic studies. Decreased insulin stimulation of glucose transport in muscle from morbidly obese and diabetic subjects. *J. Clin. Invest.* 82:486–494.

43. Klip, A. 1987. Hexose transport across skeletal muscle sarcolemma. *In* Sarcolemmal Biochemistry. Vol. 2. A. M. Kidwai, editor. CRC Press, Boca Raton, FL. 129–153.

44. Klip, A., G. Li, and W. Logan. 1984. Induction of sugar uptake response to insulin by serum depletion in fusing L6 myoblasts. *Am. J. Physiol.* 247:E291–E296.

45. Standaert, M. L., S. D. Schimmel, and R. J. Pollet. 1984. The development of insulin receptors and responses in the differentiating non-fusing muscle cell line BC3H-1. *J. Biol. Chem.* 259:2337–2345.

46. Jackson, R. A., M. I. Hawa, J. B. Jaspan, B. M. Sim, L. DiSilvio, D. Featherbe, and A. B. Kurtz. 1987. Mechanism of metformin action in non-insulin-dependent diabetes. *Diabetes.* 36:632–640.

47. Bailey, C. J. 1988. Metformin revisited: its actions and indications for use. *Diabetic Med.* 5:315–320.

48. Wu, M. S., P. Johnston, W. H.-H. Sheu, C. B. Hollenbeck, C.-Y. Jeng, I. D. Goldfine, Y.-D. I. Chen, and G. M. Reaven. 1990. Effect of metformin on carbohydrate and lipoprotein metabolism in NIDDM patients. *Diabetes Care.* 13:1–8.

49. Frayn, K. N., and P. I. Adnitt. 1972. Effects of metformin on glucose uptake by isolated diaphragm from normal and diabetic rats. *Biochem. Pharmacol.* 21:3153–3162.

50. Frayn, K. N., P. I. Adnitt, and P. Turner. 1973. The use of human skeletal muscle in vitro for biochemical and pharmacological studies of glucose uptake. *Clin. Sci. (Lond.).* 44:55–62.

51. Bailey, C. J., and J. A. Puah. 1986. Effect of metformin on glucose metabolism in mouse soleus muscle. *Diabete Metab.* 12:212–218.

52. Mesmer, O. T., and T. C. Y. Lo. 1989. Hexose transport in human myoblasts. *Biochem. J.* 262:15–24.

53. Mizel, S. B., and L. Wilson. 1972. Inhibition of the transport of several hexoses in mammalian cells by cytochalasin B. *J. Biol. Chem.* 247:4102–4105.

54. Fantus, I. G., and R. Brosseau. 1986. Mechanism of action of metformin: insulin receptor and post-receptor effects in vitro and in vivo. *J. Clin. Endocrinol. Metab.* 63:898–905.

55. Ramlal, T., V. Sarabia, P. J. Bilan, and A. Klip. 1988. Insulin-mediated translocation of glucose transporters from intracellular membranes to plasma membranes: sole mechanism of stimulation of glucose transport in L6 muscle cells. *Biochem. Biophys. Res. Commun.* 157:1329–1335.

56. Klip, A., T. Ramlal, D. A. Young, and J. O. Holloszy. 1987. Insulin-induced translocation of glucose transporters in rat hindlimb muscles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 224:224–230.

57. Hundal, H., T. Ramlal, R. Reyes, L. A. Leiter, and A. Klip, 1992. Cellular mechanism of metformin action involves glucose transporter translocation from an intracellular pool to the plasma membrane in L6 muscle cells. *Endocrinology.* In press.

58. Klip, A., A. Guma, T. Ramlal, P. Bilan, L. Lam, and L. A. Leiter. 1992. Stimulation of hexose transport by metformin in L6 muscle cells in culture. *Endocrinology.* 130:2535–2544.

59. Calderhead, D. M., K. Kitagawa, G. E. Lienhard, and G. W. Gould. 1990. Translocation of the brain-type glucose transporter largely accounts for insulin stimulation of glucose transport in BC3H-1 myocytes. *Biochem. J.* 269:597–601.

60. Mitsumoto, Y., E. Burdett, A. Grant, and A. Klip. 1991. Differential expression of the GLUT1 and GLUT4 transporters during differentiation of L6 muscle cells. *Biochem. Biophys. Res. Commun.* 175:652–659.

61. Matthaei, S., H. A. Hamann, H. H. Klein, H. Benecke, G. Kreymann, J. S. Flier, and H. Greten. 1991. Association of metformin's effect to increase insulin-stimulated glucose transport with potential of insulin-induced translocation of glucose transporters from intracellular pool to plasma membrane in rat adipocytes. *Diabetes.* 40:850–857.

62. Kahn, B. B., L. Rossetti, H. F. Lodish, and M. J. Charron. 1991. Decreased in vivo glucose uptake but normal expression of GLUT1 and GLUT4 in skeletal muscle of diabetic rats. *J. Clin. Invest.* 87:2197–2206.

63. Handberg, A., L. Kayser, P. E. Hoyer, and J. Vinten. 1991. Localization of glucose transporters GLUT1 and GLUT4 in rat skeletal muscles. *Proc. Sat. Symp. Diabetes Mellitus & Exercise.* Burlington, VT. 14.