Glucose uptake and metabolism by cultured human skeletal muscle cells: rate-limiting steps

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Glucose uptake and metabolism by cultured human skeletal muscle cells: rate-limiting steps. Am J Physiol Endocrinol Metab 281: E72–E80, 2001.—To use primary cultures of human skeletal muscle cells to establish defects in glucose metabolism that underlie clinical insulin resistance, it is necessary to define the rate-determining steps in glucose metabolism and to improve the insulin response attained in previous studies. We modified experimental conditions to achieve an insulin effect on 3-O-methylglucose transport that was more than twofold over basal. Glucose phosphorylation by hexokinase limits glucose metabolism in these cells, because the apparent Michaelis-Menten constant of coupled glucose transport and phosphorylation is intermediate between that of transport and that of the hexokinase and because rates of 2-deoxyglucose uptake and phosphorylation are less than those of glucose. The latter reflects a preference of hexokinase for glucose over 2-deoxyglucose. Cellular NAD(P)H autofluorescence, measured using two-photon excitation microscopy, is both sensitive to insulin and indicative of additional distal control steps in glucose metabolism. Whereas the predominant effect of insulin in human skeletal muscle cells is to enhance glucose transport, phosphorylation, and steps beyond, it also determines the overall rate of glucose metabolism.

transport-metabolism coupling; glucose transport; hexokinase; rate-determining step; substrate specificity

SKELETAL MUSCLE ACCOUNTS FOR as much as 80% of insulin-sensitive glucose metabolism and a substantial portion of whole body glucose metabolism in humans (1, 10). Therefore, an understanding of the mechanism and regulation of muscle glucose utilization is necessary to determine the nature of the defects present in metabolic diseases such as obesity and insulin-resistant diabetes. Glucose transport, the initial step in glucose utilization, is often considered rate determining in glucose utilization in muscle (15). However, there is evidence from clinical studies (3, 17, 30) and from studies of cultured human muscle cells (14) that glucose phosphorylation by hexokinase also affects the rate of glucose utilization, especially under conditions of high glucose concentrations or stimulation by insulin. Because vertebrate muscle has little glucose-6-phosphatase activity (28), the phosphorylation of glucose is irreversible in this tissue. Irreversibility is a feature of rate-determining steps in metabolism (22) and supports the notion that phosphorylation aids in determining the overall rate of glucose utilization. Nevertheless, the relative contributions of transport and phosphorylation as rate-determining steps in glucose utilization are difficult to ascertain.

One approach to assessing the balance of transport and phosphorylation in determining glucose utilization is to establish how these processes are coupled at the cellular level. Multiple factors determine which step is rate limiting under any given set of conditions, including the kinetics of each step, responsiveness to stimulation or feedback inhibition by glucose 6-phosphate, the intracellular glucose concentration, and the peculiar characteristics of the cell type under study. Of relevance to assessing human muscle glucose metabolism are studies using cultured human skeletal muscle cells (HSMC), derived from muscle precursor “satellite” cells. These cells are readily established in primary culture (2) and retain both insulin responsiveness (4, 13, 27) and defects in glucose utilization present in the original donor (4, 13). Most studies in these cells have used the uptake and phosphorylation of 2-deoxyglucose to follow glucose metabolism. Use of this glucose derivative allows measurement of coupled transport and phosphorylation, since 2-deoxyglucose is phosphorylated but not further metabolized by cells. However, 2-deoxyglucose is phosphorylated less well than glucose (12), and at high concentrations it can affect cellular metabolism by acting as a sink to deplete the cells of ATP. Jacobs et al. (14) found that intracellular 2-deoxyglucose accumulated during uptake into cultured human muscle cells, leading them to conclude that phosphorylation, rather than transport, is the rate-determining step in these cells. This result con-
trasts with the observations of D’Amore and Lo (9) in
the L6 line of rat skeletal muscle cells; they found that
2-deoxyglucose did not accumulate during uptake and
concluded that transport was rate limiting. Thus there is
controversy regarding the rate-limiting step for 2-de-
oxyglucose transport in cultured muscle cells, and
there is no information regarding the rate-limiting step
for glucose, the natural substrate.

Achieving adequate insulin responsiveness is crucial
to the use of primary cultures of HSMC to study defects
in insulin action at the cellular level. Insulin enhances
the rate of 2-deoxyglucose uptake in these cells from 20
or 30 (4, 5, 27) to 70% (13) above basal rates. This is
substantially less than the twofold insulin responsive-
ness typically observed in human forearm muscle bal-
ance studies (30). This low degree of insulin responsi-
veness makes these cells problematic for assessing
insulin effects on the rate-limiting step of glucose uti-
lization and for establishing the mechanism(s) im-
paired in insulin resistance.

In the present studies, we were able to consistently
achieve two- to threefold stimulation by insulin of both
transport and coupled transport-phosphorylation. We
used these cells to evaluate the rate-determining step(s)
in glucose utilization by comparing transport and
phosphorylation in the presence and absence of
insulin over the physiological range of glucose concen-
trations. The results show that insulin stimulates
transport, but phosphorylation and downstream me-
tabolic steps also determine the overall rate of glucose
utilization in these cells.

MATERIALS AND METHODS

Materials. Skeletal muscle cell growth medium (SkGM)
Bullet Kit (basal medium, insulin, fetuin, bovine serum al-
bumin (BSA), human epidermal growth factor, dexametha-
sone, gentamicin, and amphotericin B) and subculture re-
agent pack (HEPES-buffered saline solution, trypsin/EDTA
solution, and trypsin neutralizing solution) were purchased
from Clonetics (San Diego, CA). All other cell culture mate-
rials were purchased from Gibco (Grand Island, NY). The
fluorescein isothiocyanate-conjugated sheep antimalar IgG
and monoclonal anti-troponin T were obtained from Sigma
(St. Louis, MO). Monoclonal antibody IgG2b, kappa light-
chain myosin (MF20) was obtained from the Developmental
Studies Hybridoma Bank (University of Iowa, Iowa City, IA).
The creatine phosphokinase (CPK) diagnostic assay kit was
obtained from Euroclone (Waltham, MA). The 20-kDa
aminoxyanilide was purchased from Molecular Probes (Eugene,
OR). The QAE-Sephadex anion exchange resin were obtained from
Bio-Rad Laboratories, (Hercules, CA). The QAE-Sephadex anion exchange
resin was purchased from Pharmacia Fine Chemicals (Piscata-
way, NJ). All other chemicals and reagents were purchased
from Sigma-Aldrich (St. Louis, MO).

HSMC cultures. Informed consent procedures were ap-
proved by the Vanderbilt Institutional Review Board. Surgi-
cal biopsies were obtained from the sternoclomastoid mus-
cle of male and female subjects aged 35–65 yr who had
undergone thyroid or parathyroid surgery. The subjects had
no known disorders of glucose or thyroid hormone metabo-

lism. In rabbits, this muscle is composed of a mixture of fiber
types I and II (21).

Cell culture methods were those of Blau and Webster (2),
as modified by Henry et al. (13), with further modifications as
noted below. Tissue samples of 50–500 mg were placed im-
mediately in Ham’s F-12 medium at 4°C. As much connective
tissue as possible was removed from the muscle, which was
then minced and rinsed 3 times in Ham’s F-12 medium at a
temperature of 4°C, followed by a rinse in the same medium
at 37°C. Dissociation of muscle cells was accomplished by
three successive 20-min incubations in a Wheaton trypsiniza-
tion flask with 15 ml of 0.05% trypsin-EDTA and constant
agitation at 37°C. After each dissociation, the cells were
strained using a 100-μm cell strainer and kept at 4°C. At
the end of each treatment, protease activity in the filtrate was
stopped with 10% horse serum. The pooled cell suspension
was then strained again using a 40-μm cell strainer to re-
move any remaining clumps of tissue. The cell suspension
was centrifuged at 2,000 rpm for 10 min at 4°C. Isolated
satellite cells (~10^6–10^7) were suspended in SkGM (Clonetics
SkGM Bullet Kit without insulin, containing 2% fetal bovine
serum) and seeded into 100-mm dishes that had been previ-
ously coated with mouse type IV collagen. Seeding density
was 10–200 satellite cells/cm^2. Collagen-coated dishes were
prepared by incubation of 9 μg collagen/ml in Hanks’ basic
salt solution without added calcium or magnesium salts (5.0
mM KCl, 0.3 mM KH₂PO₄, 138 mM NaCl, and 0.3 mM
NaH₂PO₄, pH 7.4) at 37°C for 1 h, followed by removal of the
buffer before cell plating. Cell culture was carried out in a
humidified atmosphere of 95% air-5% CO₂ at 37°C. Media
were changed initially at 6–7 days and thereafter every 2–3
days until 50–90% confluence was attained (~4–8 wk).

First-passage cells were seeded at a density of 3,000 cells/
cm² in either 48-well tissue culture dishes for assay of
3-O-methylglucose transport and coupled transport-phos-
phorylation or 100-mm dishes for the hexokinase assays.
For the fluorescence monitoring of NAD(P)H levels by two-photon
excitation microscopy, the cells were seeded into glass-bot-
tom 35-mm dishes (PG35G, 0–14 g, Mattek, Ashland, MA).
For staining of skeletal muscle cells for specific markers, the
cells were seeded into chamber/culture slides. Once the cells
had attained >80% confluence in the growth medium, this
was replaced with fusion media, and culture was continued
for 5 days. The fusion medium consisted of aMEM, 5.6 mM
D-glucose, 2% fetal bovine serum, and 1% antibiotic/anti-
mycotic solution.

Differentiation into myotubes was confirmed by a fivefold
increase in CPK activity relative to that of unfused myoblasts
and by visualization under phase-contrast microscopy of
elongated, multinucleated cells. Preliminary antibody stain-
ing studies also confirmed that 100% of cells were HSMC.
Antibody staining was carried out by a modification of the
procedure supplied by Clonetics. Fused myotubes were fixed
in 4% formaldehyde in phosphate-buffered saline (PBS, 140
mM NaCl, 12.5 mM NaH₂PO₄, pH 7.4) for 15 min at room
temperature, permeabilized with 0.1% Triton X-100 in PBS
for a few seconds at room temperature, and then blocked with
5% sheep serum in PBS for 90 min at room temperature.
After being washed with PBS, the cells were incubated over-
night at 4°C with either a monoclonal antibody to IgG2b,
kappa light-chain myosin or a monoclonal antibody to mouse
sarcomeric troponin T. The incubations were diluted (vol/vol)
1:10 and 1:100, respectively, in 0.1% Triton X-100 in PBS.
Cells were then washed with 0.1% Triton X-100 in PBS and
incubated with the secondary antibody at room temperature
for 2 h (fluorescein isothiocyanate-conjugated sheep anti-
mouse IgG, 1:100 in 0.1% Triton X-100 in PBS). Cells were
mounted according to the manufacturer’s directions for the Slowfade AntiFade Kit (Molecular Probes, Eugene, OR) and viewed by confocal fluorescence microscopy. Fused myotubes stained in this manner were elongated, multinucleated, and showed striations characteristic of skeletal muscle cells.

Transport of 3-O-methyl-D-[14C]glucose. Assay of 3-O-methyl-D-[14C]glucose transport was carried out after a 4-h incubation of cells in α-MEM that contained 5.6 mM glucose and 0.1% BSA (fraction V) in a humidified atmosphere of 95% air-5% CO2 at 37°C. The medium was changed to modified Krebs-Ringer-HEPES buffer (KRH; 0.85 mM CaCl2, 24 mM HEPES, 5 mM glucose, 0.1% BSA, and 5 mM sodium pyruvate) in the presence or absence of 500 nM insulin, as noted. Transport was typically measured after a 1-min incubation at 37°C in KRH buffer that contained 0.2 μCi of 3-O-methyl-D-[14C]glucose, 0.5 μCi of L-[2-3H(N)]glucose, 5 mM d-glucose, 0.1 mM unlabeled 3-O-methylglucose, and 0.1 mM unlabeled d-glucose. The reaction was stopped with four rapid washes of the cells with 1 mM MgCl2 in saline (4°C). Cells were lysed by constant shaking for 10 min with 1 ml of 0.05 N sodium hydroxide. An aliquot of each sample was taken for protein analysis. Radioactivity was counted in a Packard CA2000 liquid scintillation spectrometer under dual-label settings and corrected for quenching. Specific 3-O-methyl-D-[14C]glucose transport was obtained by subtracting values obtained for non-carrier-mediated uptake of d-glucose from each sample.

Assay of hexokinase activity and metabolite concentration dependencies. HSMC were seeded into 100-mm dishes at near confluence and were allowed to fuse into myotubes as described above. The cells were then washed twice with cold PBS and were scraped from the dishes with a rubber policeman into 1.5 ml of ice-cold homogenization buffer (50 mM triethanolamine-HCl, 100 mM KCl, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 0.2% sodium azide, pH 7.5). The cells were disrupted by sonication for 3 s at maximal power (Heat Systems-Ultrasonics) and then cooled on ice for 15 s. The sonication/cooling steps were repeated two more times. Hexokinase activity was determined after a 15-min incubation of 5 μl of the homogenate in 20 μl of reaction buffer (50 mM Tris-HCl, 20 mM MgCl2, 100 mM KCl, 1 mM dithiothreitol, 0.01 μCi of D-[U-14C]glucose, and 0.05 μCi of 2-deoxy-D-[3H]glucose) at the indicated concentrations of the substrate/inhibitor.

The reaction mixture was applied to a 0.2-ml column of QAE-Sephadex (Pharmacia), and free hexoses were eluted with water. The labeled anionic phosphates were eluted by 10.2±0.3 mM KCl added directly to a 7-ml scintillation vial. Samples (20 μl) of the reaction buffer were also added to a 0.2-ml borate anion exchange column, and the 3H2O that had been released by the cells was eluted with 1.4 ml of water directly into a 7-ml scintillation vial. Radioactivity in the column eluates was measured by liquid scintillation counting, as described above.

Fluorescence monitoring of NAD(P)H levels by two-photon excitation microscopy. HMSC at near confluence in glass-bottom 35-mm dishes were rinsed free of fusion medium and cultured for 4 h in glucose-free DMEM containing 0% fetal bovine serum and then washed twice in reaction buffer (150 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM CaCl2, 2.5 mM NaH2PO4, 10 mM HEPES, 0.1% BSA, pH 7.4) and incubated in 2 ml of fresh reaction buffer.

NAD(P)H autofluorescence of HMSC was measured by two-photon laser scanning microscopy as previously described by Piston and Knobel (23). The stage and objective lens of the instrument were maintained at 37°C during the experiment. Images of the cells were acquired using the average of three scans of 3 s each. Glucose was added from an 80-mM stock solution to bring glucose to the indicated concentration. An image was acquired after 5 min of equilibration. This process was repeated with successive additions to bring glucose in the medium to the indicated concentration.

Other assays and statistical analysis. Protein was determined using the Bradford method (Bio-Rad) according to the manufacturer’s instructions. Results are expressed as means ± SD or ± SE, as indicated. Paired samples were compared by the paired Student’s t-test, and comparisons among multiple samples were carried out by one-way analysis of variance and post hoc testing with Dunnett’s test. Kinetic parameters were determined by nonlinear curve fitting of the Michaelis-Menten equation hyperbolic plots with the graphic analysis program FigP (Biosoft, St. Louis, MO).

RESULTS

Insulin-stimulated transport of 3-O-methyl-D-[14C]glucose. The initial transport step of glucose utilization was studied by measuring the uptake of 3-O-methyl-D-[14C]glucose, which is transported, but not further metabolized, by mammalian cells. Initial studies showed only a small effect (<30% stimulation) of insulin on transport by fused myotubes. Several maneuvers were tested for their effects on the relative insulin response, including type of growth and fusion media used in culture, length of serum starvation before assay, time of preincubation with insulin, and the effect of added pyruvic acid. Of these approaches, the most important for improving the insulin response involved avoiding the use of insulin in the culture medium, as well as...
during the 4- to 5-h serum-free preincubation, and the addition of 5–10 mM pyruvic acid during stimulation by insulin. Although incubation times with insulin were 1 h in the studies shown, maximal effects were achieved by 10 min of incubation. As shown in Fig. 1, pyruvate treatment decreased basal and increased insulin-stimulated transport, with a net effect of increasing the increment between basal and insulin-stimulated transport. Transport of 0.1–20 mM 3-O-methyl-[14C]glucose by fused myotubes is linear for several minutes in the absence of insulin but for only 1 min in the presence of insulin. For this reason, and because insulin responsiveness was maximal at early time points (Fig. 2), a 1-min incubation time was chosen for subsequent transport experiments.

The substrate dependence of 3-O-methyl-[14C]glucose uptake by fused myotubes was measured using the optimal conditions defined by the initial studies, with results shown in Fig. 3. Transport of 3-O-methyl-[14C]glucose is saturable, and insulin causes a consistent 2-fold increase in transport rates over the range of 3-O-methylglucose concentrations tested. As shown in Table 1, the apparent affinity of the transport step for this hexose is between 3 and 5 mM, with no significant difference between basal and insulin-treated cells. Thus the transport step in HSMC is responsive to changes in extracellular glucose over much of the physiological glucose concentration range.

**Assay of hexokinase activity in cellular extracts.** To compare the kinetics of the transport step with those of endogenous hexokinase, cultured HSMC were lysed, and hexokinase kinetics were measured in the extracts. For these experiments, hexokinase activity was determined using both glucose, the natural substrate, and 2-deoxyglucose. The latter is transported and phosphorylated but is not further metabolized (18).

![Fig. 1. Effect of sodium pyruvate on the uptake of 3-O-methyl-D-[14C]glucose by fused human skeletal muscle cells (HSMC). Four hours after the removal of serum, cells were incubated for 60 min in the absence (○) or presence (●) of 500 nM insulin and the indicated concentration of sodium pyruvate under the conditions noted in MATERIALS AND METHODS. Results are shown as means + SD of 4 determinations from an experiment representative of 3 that were performed.](http://ajpendo.physiology.org/)

![Fig. 2. Time course of 3-O-methyl-D-[14C]glucose uptake in fused HSMC. Incubations were carried out for the indicated times in the absence (○) or presence (●) of 500 nM insulin, as described in MATERIALS AND METHODS. Results are representative of 4 separate experiments, shown as means + SD.](http://ajpendo.physiology.org/)

![Fig. 3. Concentration response of 3-O-methyl-D-[14C]glucose uptake in fused HSMC. Transport assays were carried out at the indicated 3-O-methylglucose concentrations in the absence (○) or presence (●) of 500 nM insulin, as described in MATERIALS AND METHODS. Results are shown from 4 separate experiments as means + SE.](http://ajpendo.physiology.org/)
Glucose was used to inhibit the metabolism of tracers of both glucose and 2-deoxyglucose, so the results are shown as clearance of the respective tracers. Because glucose (and not 2-deoxyglucose) concentrations were varied, it was possible to calculate apparent kinetic constants only for glucose; these are shown in Table 1. As is evident from Fig. 4A, glucose is the preferred substrate for the hexokinase activity present in these cells. A similar relationship is observed when the concentration of ATP is varied and that of the hexose substrate is held constant, as shown in Fig. 4B. However, the ability of glucose 6-phosphate to inhibit hexose phosphorylation is similar for glucose and 2-deoxyglucose (Fig. 4C), since the inhibition constant ($K_i$) for glucose is $70 \pm 10$ μM and that for 2-deoxyglucose is $90 \pm 40$ μM. These results show that glucose is a much better substrate for human muscle cell hexokinase than is 2-deoxyglucose. Because this difference could be useful in differentiating transport from phosphorylation as the rate-determining step, we next compared glucose transport and phosphorylation of the two hexoses in intact cells.

**Assay of coupled glucose transport and phosphorylation for glucose and 2-deoxyglucose.** Steady-state glucose phosphorylation, measured as the release of $^3$H$_2$O from [2-$^3$H]glucose, is saturable with increasing medium glucose (Fig. 5A). These results are expressed as clearances to facilitate comparison with the results of glucose inhibition of 2-deoxyglucose uptake (Fig. 5B). Rates of glucose utilization derived from these data were used to calculate the kinetic parameters shown in Table 1. Over the range of glucose concentrations studied, insulin causes a twofold increase in glucose transport-phosphorylation measured by this assay but is without effect on the apparent Michaelis-Menten constant ($K_m$). In results not shown, the effect of insulin was complete by 10 min, and the minimal concentration of insulin required for a maximal effect was 50 nM. It is important to note that the apparent $K_m$ of coupled transport and phosphorylation of glucose is intermediate between that of transport and hexokinase (Table 1). That is, the assay of coupled transport and phosphorylation shows an increased sensitivity of the system to glucose, which must be due to the phosphorylation step.

Uptake and phosphorylation of 2-deoxy-[14C]glucose, expressed as a clearance, are inhibited by the presence of increasing medium glucose concentrations (Fig. 5B). Unlabeled glucose, rather than 2-deoxyglucose, was used in this experiment to generate glucose 6-phosphate to feedback-inhibit the hexokinase. It has been shown that 2-deoxyglucose 6-phosphate does not inhibit hexokinases (8), so the use of increasing concen-

![Fig. 4. Hexokinase activity in lysates of HSMC. A: glucose concentration response (at 10 mM ATP); B: ATP concentration response (at 0.2 mM D-glucose); C: glucose 6-phosphate concentration response (at 0.2 mM D-glucose and 10 mM ATP). Results are shown for the 2-[1,2-$^3$H(N)]deoxyglucose tracer (○) and for D-[U-14C]glucose tracer (■) as means ± SE from 5 or 6 independent assays.](http://ajpendo.physiology.org/)
The concentration of unlabeled glucose allows for a comparison of the effects of tracer glucose and 2-deoxy-[14C]glucose at low glucose concentrations, although this effect is not evident at higher medium glucose concentrations. Because glucose and 2-deoxyglucose are transported with similar affinities, the finding that glucose is a better substrate for the coupled transporter-hexokinase pair than is 2-deoxyglucose suggests that the hexokinase step affects glucose metabolism over a range of glucose concentrations. To further evaluate this possibility, cytochalasin B was used to make transport more rate limiting for utilization.

Cytochalasin B is a specific inhibitor of the glucose transport step (29) and thus should make transport more limiting for overall glucose metabolism. As shown in Fig. 6, in the absence of cytochalasin B, the ratio of clearances of 2-deoxyglucose and glucose is ~0.5. However, when transport is inhibited by ~50% with 0.2 μM cytochalasin B and by ~75% with 1 μM cytochalasin B, the ratio progressively increases, although not to unity. This result suggests that transport becomes more rate determining in the presence of cytochalasin B.

Fluorescence monitoring of NAD(P)H levels by two-photon excitation microscopy. To assess whether transport-phosphorylation is rate limiting for overall glucose utilization in cultured skeletal muscle cells, we used two-photon excitation microscopy to follow glucose-induced autofluorescence of NAD(P)H in the cells (24). Incubation of cells with progressively increasing concentrations of glucose leads to increases in cellular NAD(P)H autofluorescence (Fig. 7). This autofluorescence saturates in both basal and insulin-treated cells with apparent $K_m$ values of 0.4 ± 0.1 and 0.3 ± 0.05 mM, respectively. That is, at this end stage of glucose utilization, cells are very sensitive to the medium glucose.

Fig. 5. Coupled hexose transport and phosphorylation in fused HSMC. A: clearance of $^3$H$_2$O due to coupled transport and phosphorylation of $\delta$-[2-$^3$H]glucose. B: clearance of 2-[14C]deoxyglucose 6-phosphate from the coupled transport and phosphorylation of 2-[14C]deoxyglucose. Cells were incubated for 1 h in the absence (■) or presence (▲) of 500 nM insulin. Results are shown as means ± SE of 3 independent experiments.

Fig. 6. Effects of cytochalasin B on coupled hexose transport and phosphorylation in fused HSMC. Clearances of [14C]deoxyglucose 6-phosphate and $^3$H$_2$O were measured in the presence of 0.2 mM D-glucose as described in MATERIALS AND METHODS. The ratio of the clearance of [14C]deoxyglucose 6-phosphate to that of $^3$H$_2$O ($C_{\text{DG}}/C_{\text{GLC}}$) is shown as a function of the added concentration of cytochalasin B. Results are provided from 4 separate experiments as means ± SE. *$P < 0.05$ vs. no cytochalasin B.
cose concentration, and maximal effects are observed at extracellular glucose concentrations that were lower than what was observed at the transport step and less than one-half of that observed for 2-[3H]glucose transport-phosphorylation. Although not apparent in Fig. 7A because the data were normalized to the maximal autofluorescence, the addition of insulin to cells that had already been incubated in glucose caused an additional increase in NAD(P)H autofluorescence. Figure 7B shows images of the same group of cells before addition of glucose (left), after 10 min of incubation with 5 mM D-glucose (middle), and 10 min after the addition of insulin (right). After correction for the autofluorescence observed before glucose addition, insulin doubles the relative autofluorescence. Thus the insulin response is apparent at this late stage of glucose utilization.

DISCUSSION

The results of the present studies show that we were able to enhance the insulin responses obtained in primary cultures of HSMC. Preincubation of the cells in serum-free medium (27) in the absence of insulin (4) and in the presence of pyruvate (19) significantly enhances the response of the glucose transport step to insulin. Although the mechanism of the pyruvate effect is not known, the enhanced insulin action is due largely to a decrease in basal transport rates in both HSMC (Fig. 1) and rat adipose cells (19). Inclusion of pyruvate may be a useful means of obtaining maximal insulin responses in these cells, especially when the objective is to compare responses of cells derived from insulin-resistant subjects with cells from control subjects.

At each stage of glucose utilization studied, the HMSC became more sensitive to the effects of glucose. Thus the apparent $K_m$ of the transport step measured by 3-O-methylglucose uptake was 3–5 mM. When transport and phosphorylation were measured as a coupled reaction, using the release of $^3$H$_2$O from 2-[3H]glucose, the apparent $K_m$ decreased to just over 1 mM. Finally, when NAD(P)H autofluorescence was measured as an end step in glucose utilization, the apparent $K_m$ fell to $\sim$0.3–0.4 mM. The decrease in the apparent $K_m$ at the hexokinase step is due to the high affinity of hexokinases for glucose compared with that

Fig. 7. NAD(P)H autofluorescence measured by two-photon excitation microscopy. A: fused HSMC were incubated for 1 h at 37°C in the absence (c, solid line) or presence (■, dashed line) of 500 nM insulin, followed by addition of the indicated glucose concentration and image acquisition. Results are shown as means ± SE from 4–5 experiments, each on 4–7 myotubes. All results were normalized to the maximal fluorescence signal. Lines represent nonlinear fits to the Michaelis-Menten equation. B: effect of treatment with 5 mM D-glucose followed by sequential treatment with 500 nM insulin for 10 min on NAD(P)H autofluorescence in a group of HSMC.
of the transport step. The measured $K_m$ of glucose phosphorylation in cell extracts is 70 $\mu$M, which is almost two orders of magnitude less than the $K_m$ for transport (Table 1). The finding that the $K_m$ of coupled transport-phosphorylation of glucose and 2-deoxyglucose shifts downward from that of transport alone shows that the phosphorylation step does affect the overall rate of glucose utilization. The additional decrease in $K_m$ observed in the autofluorescence studies suggests that steps beyond the hexokinase are also important in determining the rate at which glucose is metabolized. Our finding that the maximal rate of glucose phosphorylation by cell extracts greatly exceeds coupled transport and phosphorylation (Table 1) suggests that hexokinase activity is controlled in the cell. This might be due to limited substrate availability, to compartmentalization within cells, or to feedback inhibition of the hexokinase by accumulated glucose 6-phosphate.

Differences in the specificity of transport and hexokinase for glucose and 2-deoxyglucose provide additional evidence that phosphorylation is rate limiting for glucose utilization in HSMC. Both hexoses are transported equally well by the GLUT-type transporters present in these cells (11, 20). However, 2-deoxyglucose is a much poorer substrate for hexokinase than is glucose (Ref. 12 and Fig. 4A of this work), an effect that is also present at varying ATP concentrations (Fig. 4B). A similar specificity has been observed previously for these cells (14). The finding that 2-deoxyglucose is transported and phosphorylated at one-half the rate of glucose over a range of medium glucose concentrations, whether or not insulin is present (Fig. 5), indicates that phosphorylation is important in determining the overall rate of glucose utilization. This preference for glucose over 2-deoxyglucose persists even when the transport rate is selectively decreased by cytochalasin B (Fig. 6). However, as the cytochalasin B concentration is increased, the ratio of 2-deoxyglucose to glucose clearance ($C_{2DG}/C_{GLC}$) increased to 0.72. Under these conditions transport has become more rate determining for overall glucose metabolism. Jacobs et al. (14) found that rates of transport of 2-deoxyglucose in cultured HSMC greatly exceeded rates of phosphorylation, leading to the accumulation of unphosphorylated 2-deoxyglucose. They concluded that phosphorylation, rather than transport, is rate limiting for glucose metabolism. Our conclusion, based on the results presented, is in agreement with that of Jacobs et al.

These findings have relevance for the insulin resistance in muscle that contributes to the development of non-insulin-dependent diabetes and is associated with obesity. Much effort has been expended in an attempt to determine the location of the defect responsible for insulin resistance in vivo. Glucose transport and phosphorylation have both been implicated in the defective glucose utilization noted in type 2 diabetes mellitus. Bonadonna et al. (3) investigated nondiabetic controls and patients with type 2 diabetes mellitus by use of forearm balance techniques and euglycemic glucose clamps. They found that glucose transport was decreased ~40% but that the combination of transport and phosphorylation was decreased 85% in type 2 diabetic patients. When the plasma glucose concentration in the diabetics was raised to 13 mM, rates of transport of the nonmetabolized 3-O-methyl-D-[1-14C]glucose were normalized, but rates of glucose phosphorylation remained less than one-half those observed in the nondiabetic controls (3). Moreover, the estimated intracellular glucose concentration was 2.5-fold higher in the diabetics than in controls under the euglycemic conditions and did not decrease with insulin infusion. Measurement of muscle glucose metabolism by means of magnetic resonance imaging in type 2 diabetes mellitus patients showed initially that the insulin resistance defect lies at the level of transport-phosphorylation (6, 25, 26), whereas most recent results locate the defect at the transport step (7). The reconciliation of these apparently disparate results may be that control is exerted at several steps and that this may change according to the metabolic circumstances and the immediate cellular environment. Although results from cultured cells must be extrapolated to the in vivo setting with caution, our results support the notion that phosphorylation and steps beyond limit glucose utilization. It will be interesting to use the approaches developed in these studies to compare glucose transport-phosphorylation in muscle cells derived from insulin-resistant persons with diabetes and those in cells from nondiabetics.

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