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Cellular mechanism of the insulin-like effect of growth hormone in adipocytes

Rapid translocation of the HepG2-type and adipocyte/muscle glucose transporters

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The cellular mechanism whereby growth hormone (GH) acutely stimulates adipocyte glucose uptake was studied in cultures of primary rat adipocytes differentiated *in vitro*. Preadipocytes were isolated by collagenase digestion of inguinal fat-pads from young rats and were differentiated in the presence of 3-isobutyl-1-methylxanthine, insulin and dexamethasone. The development of an adipocyte morphology (i.e. lipid inclusions) was observed over 6 days after initiation of differentiation. Coincident with this phenotypic change was an increase in glyceraldehyde-3-phosphate dehydrogenase (GPDH) activity and in cellular content of the HepG2-type (Glut1) and adipocyte/muscle (Glut4) glucose transporter isoforms as determined by Western immunoblotting of total cellular protein. Age-matched undifferentiated cells expressed the Glut1 transporter and low levels of GPDH, but neither accumulated lipid nor exhibited measurable expression of the Glut4 protein. On day 6 after the initiation of differentiation, GH and insulin stimulated 2-deoxy[^{14}C]glucose uptake in a dose- and time-dependent fashion in adipocytes cultured under serum-free conditions for at least 15 h. Western-blot analysis of subcellular fractions revealed that both GH and insulin rapidly (within 20 min) stimulated translocation of the Glut1 and Glut4 proteins from a low-density microsomal fraction to the plasma membrane. Confirmatory evidence was provided in immunocytochemical experiments utilizing antisera directed against the *C*-terminal region of the Glut4 protein and a fluorescein isothiocyanate-labelled second antibody. Observation of the cells via confocal laser microscopic imaging was consistent with glucose transporter redistribution from an intracellular region to the plasma membrane after treatment with GH or insulin. On the basis of these data, we suggest that the insulin-like effect of GH on adipocyte glucose transport involves translocation of the Glut1 and Glut4 proteins to the plasma membrane. Furthermore, stimulation of glucose-transporter translocation by both GH and insulin may indicate a common cell signalling element between the adipocyte GH and insulin receptors or, alternatively, the existence of multiple cellular mechanisms for stimulating glucose-transporter translocation.

INTRODUCTION

Elucidating the role of growth hormone (GH) in animal growth and development has been the goal of endocrinologists since Evans & Long [1] observed that injections of crude hypophysial extracts promoted the growth of rats. Purification of the GH molecule [2] and the molecular cloning of the GH cDNA [3] have permitted extensive investigations into the effects of GH on physiological processes. Less is known, however, about specific cellular mechanism(s) subserving the effects of GH. The discovery that insulin-like growth factor-I (IGF-I) mediates some aspects of GH action [4] and that IGF-I synthesis is regulated by GH [5] established the detection of IGF-I mRNA accumulation and/or IGF-I synthesis as evidence of a GH-induced effect [6]. It has been demonstrated that GH also directly affects cellular growth processes independent of any action of IGF-I [7]. Such effects are often subtle and, like the induction of IGF-I gene expression, are distal to initial events after activation of the GH receptor. The lack of a rapid measurable cell response to GH-receptor activation has hindered efforts to understand the cell signalling apparatus of this receptor.

The effects of GH on protein [8], carbohydrate [9] and lipid [10] metabolism in the whole animal suggest that various tissues are responsive to GH. Radio-receptor assays and the recent cloning of the GH-receptor cDNA [11–14] have provided additional evidence supporting the concept of numerous GH target cells, including adipocytes. The effects of GH on adipocyte metabolism include increased lipolysis [15], decreased glucose utilization [16] and modified glucose transport [17] evoking hyperlipidaemia, hyperglycaemia and hyperinsulinaemia observed under conditions of GH excesses *in vivo*. Most curious of the effects of GH on adipocytes are the acute insulin-like and chronic anti-insulin effects. Specifically, adipocytes deprived of GH respond acutely to GH stimulation in an insulin-like fashion (i.e. increased glucose transport) [17], whereas chronic GH treatment inhibits insulin-stimulated glucose transport [18].

Facilitative glucose transport [19,20] occurs via membrane transport proteins [21–26] which permit the bidirectional diffusion of glucose across the plasma membrane of the cell. In preadipocytes, an erythrocyte-type glucose transporter (Glut1) protein provides basal glucose transport, whereas fully differentiated adipocytes express an additional glucose-transporter isoform, the adipocyte/muscle glucose transporter (Glut4) [28]. The Glut4 transporter, localized in an intracellular membrane pool, undergoes rapid translocation to the plasma membrane upon stimulation of the cell by insulin. To a lesser extent, insulin also

Abbreviations used: GH, growth hormone; bGH, bovine GH; Glut1 and Glut4 designate the HepG2-type and adipocyte/muscle glucose transporters respectively, according to the nomenclature of Fukumoto *et al.* [27]; DME, Dulbecco's modified Eagle's medium; FBS, fetal-bovine serum; KRP, Krebs–Ringer phosphate; PBS, phosphate-buffered saline; LDM, low-density microsomes; IGF-I, insulin-like growth factor-I; IBMX, 3-isobutyl-1-methylxanthine; MID, insulin/IBMX/dexamethasone; GPDH, glycerol-3-phosphate dehydrogenase (EC 1.1.1.8).

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stimulates Glut1 translocation in adipocytes. Because the acute effect of GH on glucose transport in adipocytes resembles the effect of insulin, we surmised that a similar cellular mechanism (i.e. glucose-transporter translocation) might be involved in the GH effect. We report that GH stimulates adipocyte glucose uptake via the rapid translocation of the Glut4 and Glut1 proteins to the plasma membrane. We have identified a rapid and measurable cellular response to activation of the GH receptor, as well as a target molecule which mediates this effect. These findings may facilitate the elucidation of cellular mechanisms subserving the GH receptor.

MATERIALS AND METHODS

Cell culture

Weaned Sprague–Dawley rats (approx. 75 g body wt.; $n = 20$ per study) were housed overnight (12 h-light/12 h-dark cycle) with food and water after receipt from the vendor. Inguinal fat-pads were aseptically removed from rats immediately after killing, minced finely with scissors and scalpel and placed in sterile collagenase digestion buffer [pH 7.4; 100 mM-Hepes, 128 mM-NaCl, 10 mM-$CaCl_2$, 5 mM-dextrose, 1.5% (w/v) BSA (A 7888; Sigma, St. Louis, MO, U.S.A.), penicillin (100 units/ml)/streptomycin (100 μg/ml) (Gibco, Grand Island, NY, U.S.A.) containing 0.15% (w/v) type 1 collagenase (Worthington, Freehold, NJ, U.S.A.)]. Adipose tissue was digested for 1 h with agitation at 37 °C and then centrifuged at 200 *g* for 5 min. The supernatant containing differentiated adipocytes was discarded and the cell pellet was resuspended in Dulbecco's modified Eagle's medium (DME; Gibco). The cell suspension was then filtered through two layers of sterile gauze to remove debris, and viable cell number was determined by fluorescein diacetate staining [29]. Approx. $(2–3) \times 10^7$ viable cells were obtained per 20 rats. Cells were resuspended in DME containing 10% (v/v) fetal-bovine serum (FBS; Sigma), 2 mM-L-glutamine (Gibco), 100 units of penicillin/ml and 100 μg of streptomycin/ml at 10^5 viable cells/ml. A 2 ml portion of the cell suspension was used to inoculate each 35 mm-diam. culture dish (Falcon, Oxnard, CA, U.S.A.) (for glucose transport experiments) or approx. 6 ml for each 100 mm-diam. culture dish (for subcellular-fractionation experiments). Cells were cultured in a humidified atmosphere of air/CO_2 (19:1) at 37 °C. After the cells reached confluence (usually 3–4 days), the culture medium was replaced with MID medium [DME + 10% FBS as above supplemented with 670 nM pig insulin (Lilly, Indianapolis, IN, U.S.A.), 0.5 mM-3-isobutyl-1-methylxanthine (IBMX; Sigma) and 25 nM-dexamethasone (Sigma)] for 48 h. MID medium was replaced with fresh culture medium without added insulin, IBMX or dexamethasone for an additional 72–84 h. No attempt was made to optimize media components which maximize preadipocyte differentiation in our system. Instead, we have found that the differentiation regimen used in the conversion of 3T3-L1 cells (i.e. 48 h treatment with MID) in our laboratory [16] routinely provides approx. 70–80% conversion of rat preadipocytes into adipocytes. The developing cells exhibit characteristics typical of adipocytes, but are amenable to prolonged treatment and maintenance in culture.

Measurement of glycerol-3-phosphate dehydrogenase (GPDH)

Cells were cultured and differentiated in 35 mm culture dishes as described above. After induction of differentiation, two dishes of cells from MID-treated (induced) or age-matched non-MID-treated (non-induced) cells were harvested daily in ice-cold Lysate Buffer [25 mM-Tris/HCl (Sigma)/1 mM-EDTA (Sigma), pH 7.5]. The cells were ultrasonically disrupted with a model W-375 sonicator (Heat Systems–Ultrasonics, Farmingdale, NY, U.S.A.), centrifuged in a micro-centrifuge to remove cell debris, and then stored at −70 °C. Enzyme activity was determined conducted as previously described [30].

Glucose uptake

For a period of at least 15 h before glucose-uptake or subcellular-fractionation experiments, cells were cultured in DME with 1% BSA, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cell monolayers (35 mm dishes) were washed with Krebs–Ringer phosphate (KRP; 128 mM-NaCl, 4.7 mM-KCl, 1.25 mM-$CaCl_2$, 1.25 mM-$MgSO_4$, 10.0 mM-NaH_2PO_4/Na_2HPO_4, pH 7.4) and then equilibrated in KRP at 37 °C for 10 min before addition of hormones. Recombinant bovine GH (bGH; Monsanto Co., St. Louis, MO, U.S.A.) or insulin was then added to the culture dishes to give the appropriate final concentration, such that each dish contained an equivalent total volume of KRP and hormone. After exposure of the cells to bGH or insulin for the prescribed periods, 0.2 mM-2-[^{14}C]deoxyglucose (0.3 Ci/mmol) was added to the culture dishes for a further 10 min. Glucose uptake was linear for at least 10 min. The cells were then washed with ice-cold Dulbecco's phosphate-buffered saline (PBS) dissolved in 0.5 M-NaOH containing 0.1% (w/v) SDS, and radioactivity in each sample was measured in a Beckman liquid-scintillation counter. Non-carrier-mediated uptake was assessed in the presence of 40 μM-cytochalasin B (Sigma). All measurements were done at least in duplicate, and calculations are normalized for protein content.

Cell fractionation

Cells used in fractionation experiments were cultured in 100 mm culture dishes as described above. Cells were incubated in 1% (w/v) BSA/DME for at least 15 h before experiments and were equilibrated in KRP before hormone challenge as for the glucose-uptake experiments. After the 20 min hormone treatment period, cell monolayers were quickly washed in ice-cold HES (50 mM-Hepes, 1 mM-EDTA, 250 mM-sucrose, pH 7.4) and then removed from the dish in HES buffer with a cell scraper. Typically, 6–8 dishes were pooled within each treatment per experiment. The cell suspension was homogenized in a 55 ml glass homogenizer with Teflon pestle (Wheaton Instruments, Millville, NJ, U.S.A.) with a Wheaton Overhead Stirrer at setting 6. Cell fractions were obtained by differential centrifugation as described previously [31]. All fractions were resuspended in HES, assayed for protein content by the bichinchoninic acid procedure (Pierce, Rockford, IL, U.S.A.) and frozen at −20 °C.

Western blotting of glucose transporter proteins

For immunodetection of total cellular glucose transporter protein, cell monolayers were solubilized in ice-cold homogenization buffer containing 10 mM-Tris (pH 7.5), 150 mM-NaCl, 0.5% (w/v) Triton X-100 (Bio-Rad, Richmond, CA, U.S.A.), 0.5% sodium deoxycholate and 1.0 mM of each of the following proteinase inhibitors (Sigma): phenylmethanesulphonyl fluoride, tosyl-lysylchloromethane and tosylphenylalanylchloromethane. Total cellular homogenate (50 μg) or subcellular fractions (10–20 μg), obtained as described above, were subjected to SDS/PAGE electrophoresis (10%-acrylamide resolving gel). Proteins were electrophoretically transferred to nitrocellulose membranes. Polyclonal antisera R820 (Glut4) or R493 (Glut1) [25] were used as primary antibody at a 1:500 dilution. Membranes were incubated with R820 antisera (in PBS) for 1 h and then washed three times (15 min each) with PBS + 1% Triton X-100. Immunoreactive Glut4 was detected with ^{125}I-labelled donkey anti-rabbit IgG (Amersham, Arlington Heights, IL, U.S.A.) and was used at 0.2 μCi per membrane. After 1 h of incubation, the membranes were washed three times in PBS + 1% Triton X-100 and exposed to film with intensifying screens

(DuPont Cronex) at −70 °C. Whole-band quantification was accomplished by densitometric scanning of autoradiograms (BioImage Visage 2000, Ann Arbor, MI, U.S.A.) or by nitrocellulose-filter phosphor-imaging (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Estimation of cellular glucose-transporter isoform level

The absolute amounts of Glut1 and Glut4 proteins in differentiated adipocytes were determined by immunoblotting whole-cell homogenates (harvested in homogenization buffer described in the previous section) titrated against either purified erythrocyte glucose transporter (kindly provided by Dr. G. E. Lienhard, Department of Biochemistry, Dartmouth Medical School) or rat adipocyte low-density microsomes (LDM). The Glut4 protein content of adipocyte LDM (60 pmol/mg of protein) was determined by measuring the number of D-glucose-inhibitable cytochalasin B-binding sites essentially as previously described [19]. Approx. 90% of the cytochalasin B-binding sites in the LDM are Glut4 [32,33]. Total cellular protein (50 μg) and appropriate amounts of erythrocyte transporter or LDM (to construct a standard curve) were subjected to SDS/PAGE and immunoblotted as detailed in the previous section. Whole-band quantification was accomplished by phosphor-imaging of the nitrocellulose filters.

Immunocytochemistry

For the confocal immunocytochemistry experiments, cells were plated, at the density described above, into 35 mm culture dishes containing sterile untreated 22 mm^2 glass coverslips (Corning). Before reaching confluence, cells were differentiated as described above and were used on day 6 after addition of differentiation medium. Cells were incubated in DME + 1% (w/v) BSA for at least 12 h before treatment with bGH (4.5 nM) or insulin (100 nM) for 20 min. After the hormone challenge, the cells were washed with PBS and the medium was replaced with DME + 4% (w/v) paraformaldehyde (pH 7.4) for 20 min. Cells were then washed with PBS and incubated with 100 mM-glycine in PBS for an additional 10 min. The cells were washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min and then incubated in 2% (v/v) horse serum (Hazelton) for several hours. The cells were then washed three times with PBS and then incubated with F349 (Glut4) antiserum (1:250 dilution) in PBS containing 0.2% horse serum. After several washes with PBS, 1.5 μg of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Cappel, West Chester, PA, U.S.A.) was added to each coverslip for 1 h, and excess was removed by three washes with PBS. The coverslips were mounted on to microscope slides in PBS with 1% (w/v) n-propyl gallate and 50% (v/v) glycerol.

Imaging was conducted with a Zeiss Axioplan microscope equipped with a Bio-Rad MRC-500 laser confocal imaging system. All images were digitally enhanced and scaled by using software from the imaging system. Specificity of antisera was demonstrated by lack of fluorescence in the absence of primary antibody.

Statistics

Where appropriate, Student's t-test was used for data analysis to determine statistical significance.

RESULTS

Development of adipocyte morphology and expression of glucose transporter proteins

After reaching confluence, cultured rat preadipocytes were induced to differentiate by addition of IBMX, insulin and dexamethasone (MID) to the culture medium for 48 h as

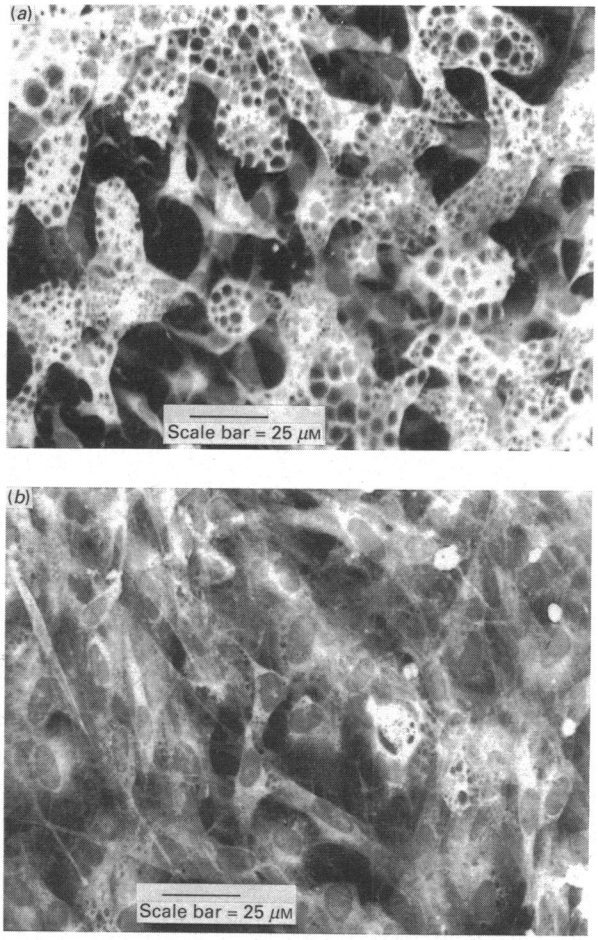

Fig. 1. Changes in cellular morphology during MID-induced differentiation

Preadipocytes were grown to confluence on glass coverslips and incubated in DME + 10% FBS (*a*) with (MID) or (*b*) without (no MID) 670 nM pig insulin, 0.5 mM-IBMX and 25 nM-dexamethasone for 48 h. Media was then replaced with DME + 10% FBS in all cultures. A representative coverslip of MID-induced and age-matched undifferentiated cells was fixed in 4% paraformaldehyde on day 4 after removal of MID.

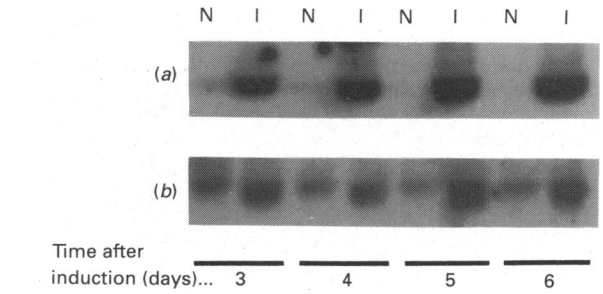

Fig. 2. Expression of Glut4 and Glut1 transporter proteins during adipocyte conversion

Preadipocytes were grown to confluence in 35 mm culture dishes and incubated in DME + 10% FBS with (induced; I) or without (non-induced; N) 670 nM pig insulin, 0.5 mM-IBMX and 25 nM-dexamethasone (MID) for 48 h. Media were then replaced with DME + 10% FBS in all cultures. Total cellular protein was extracted from cultures, and 50 μg was subjected to SDS/PAGE in a 10%-acrylamide resolving gel. Separate gels were used for immunoblot analysis of Glut4 (*a*) and Glut1 (*b*) proteins. A representative immunoblot of cellular protein from three separate platings of cells is shown.

Table 1. Acute stimulation of adipocyte glucose uptake by bGH or insulin

Cultures of adipocytes differentiated *in vitro* were incubated in DME+1% BSA for at least 15 h on day 6 after induction of differentiation. Glucose uptake was determined by measuring intracellular accumulation of 2-deoxy[^{14}C]glucose for 10 min after a 10 min incubation period with the indicated doses of bGH or insulin. Values represent means ± S.E.M., relative to control culture glucose uptake for experiments from seven independent preparations of cells. Uptake measurements were conducted at least in duplicate and have been normalized for protein content. Non-carrier-mediated uptake has been subtracted.

Treatment	Glucose uptake (% of control)
Control	100
GH (nM)	
0.0045	116 ± 21
0.045	125 ± 31
0.45	191 ± 64
4.5	354 ± 130 ($P < 0.05$)
Insulin (nM)	
0.1	110 ± 20
1	200 ± 76
10	410 ± 120 ($P < 0.05$)
100	460 ± 170 ($P < 0.05$)

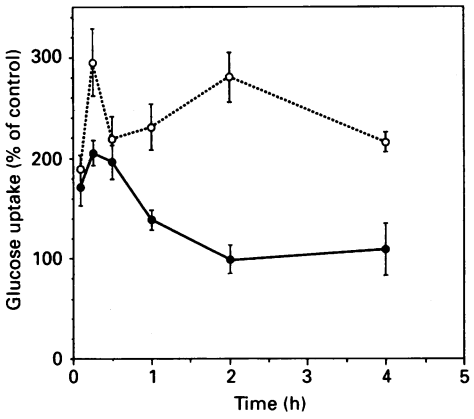

Fig. 3. Time course of bGH- or insulin-stimulated adipocyte glucose uptake

Cultures of adipocytes differentiated *in vitro* were incubated in DME+1% BSA for at least 15 h on day 6 after induction of differentiation. Glucose uptake was determined by measuring intracellular accumulation of 2-deoxy[^{14}C]glucose for 10 min after incubation with bGH (4.5 nM; ●) or insulin (100 nM; ○) for the indicated times. Values represent means for experiments from seven independent preparations of cells. Bars represent S.E.M. Uptake measurements were conducted at least in duplicate and have been normalized for protein content. Non-carrier-mediated glucose uptake has been subtracted.

previously described [16]. MID-treated cells began to acquire an adipocyte morphology (i.e. rounded shape with lipid inclusions) within 24 h after removal of the treatment medium (Fig. 1*a*). By comparison, age-matched non-MID-treated cells retained a pre-adipocyte morphology (Fig. 1*b*) with no visible lipid inclusions. By day 4 after removal of MID, the treated cells appeared to be fully differentiated as determined by the presence of numerous lipid droplets. Typically, 70–80% of the cells exhibited this morphology. Coincident with the morphological development was a greater than 33-fold induction of GPDH enzyme activity, a marker of adipocyte differentiation [30], compared with parallel

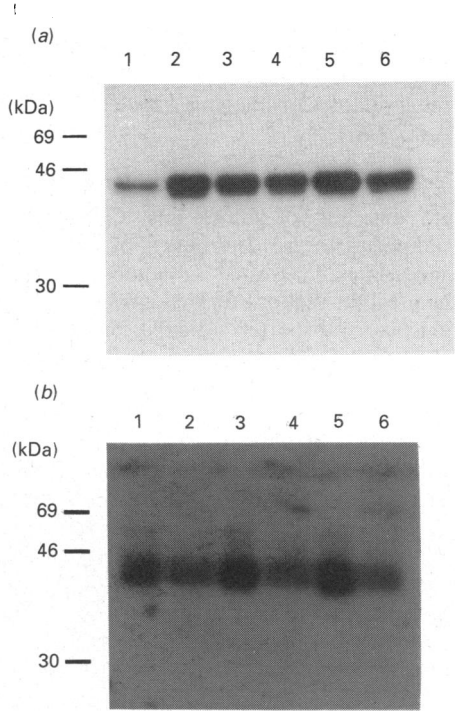

Fig. 4. Glucose-transporter translocation after acute exposure to bGH or insulin

Cultures of adipocytes differentiated *in vitro* were incubated in DME+1% BSA for at least 15 h on day 6 after induction of differentiation. After a 20 min exposure period to no treatment (lanes 1 and 2), bGH (4.5 nM; lanes 3 and 4) or insulin (100 nM; lanes 5 and 6), adipocytes were fractionated as described in the Materials and methods section. Samples (25 μg) of protein from plasma-membrane fraction (lanes 1, 3 and 5) and LDM fraction (lanes 2, 4 and 6) were subjected to SDS/PAGE in a 10%-acrylamide resolving gel and Western-blotted with antiserum to Glut4 (*a*) or Glut1 (*b*). A representative gel from four separate experiments is shown.

undifferentiated cells. The GPDH level in non-induced cells was 49 ± 20 m-units/mg of protein and increased to 1664 ± 303 m-units/mg of protein after differentiation.

Expression of Glut1 and Glut4 transporter proteins was determined by immunoblotting whole-cell protein homogenates. As depicted in Fig. 2(*a*), Glut4 protein content was markedly increased 3–6 days after MID induction, compared with the barely detectable levels of Glut4 in uninduced cells. Glut1 protein content was detectable in protein homogenates of both induced and uninduced cells. However, Glut1 protein (Fig. 2*b*) was consistently more abundant in induced cells than in uninduced cells over days 3–6 after MID treatment. The absolute amounts of Glut1 and Glut4 proteins in adipocytes 6 days after induction of differentiation were approx. 130 and 27 ng/mg of total cellular protein respectively. Glut4 protein was not detectable (< 1 ng/mg of total cellular protein) in uninduced cells 6 days after induction of differentiation. Cellular content of Glut1 protein in uninduced cells at the same state was approx. 20 ng/mg of total cellular protein.

bGH and insulin stimulation of glucose uptake

The effect of bGH and insulin on adipocyte glucose uptake was determined with adipocytes 6 days after initiation of differentiation. As described above, cells at this stage of differentiation exhibited adipocyte morphology and expression of the Glut4 protein. Both bGH and insulin stimulated 2-deoxy[^{14}C]-glucose uptake in adipocytes in a dose-related manner (Table 1).

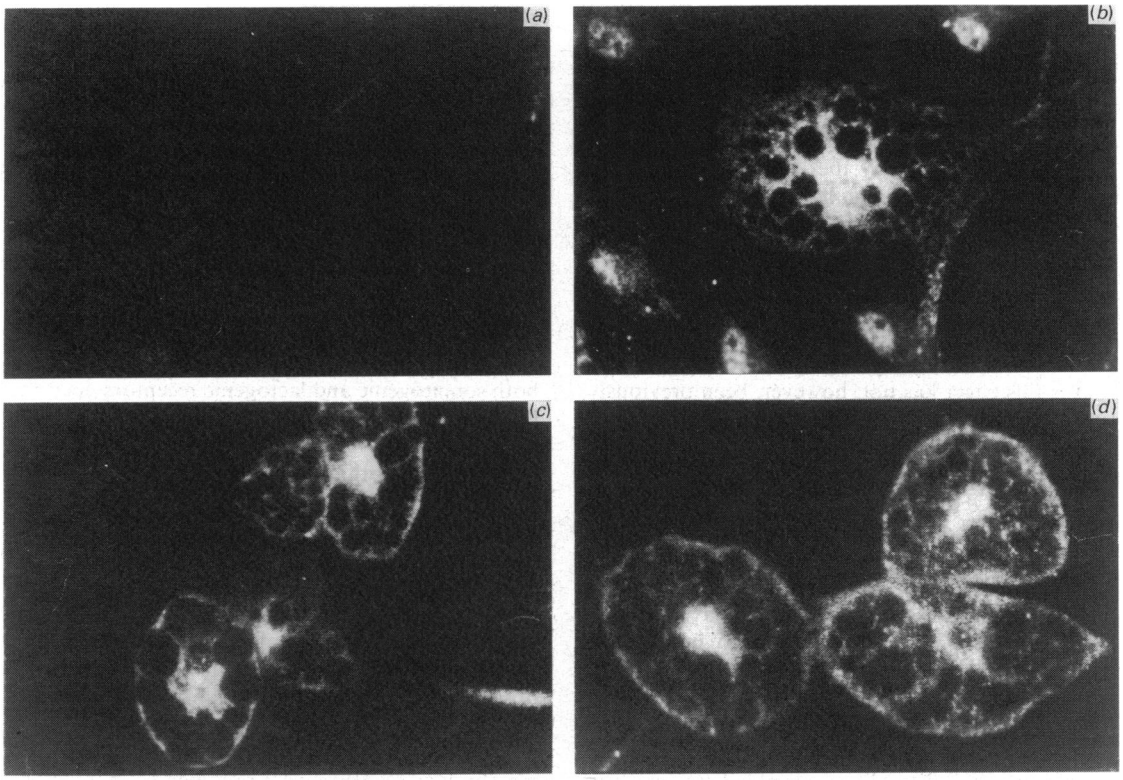

Fig. 5. Immunofluorescence confocal microscopy of acutely treated adipocytes

Preadipocytes were grown to confluence on glass coverslips and incubated in DME + 10 % FBS with (MID) or without (no MID) 670 nM pig insulin, 0.5 mM-IBMX and 25 nM-dexamethasone for 48 h. Media were then replaced with DME + 10 % FBS in all cultures. Cultures of adipocytes differentiated *in vitro* were incubated in DME + 1 % BSA for at least 15 h on day 6 after induction of differentiation. After a 20 min exposure to no hormone (control), bGH (4.5 nM) or insulin (100 nM), adipocytes were processed for Glut4 detection as described in the Materials and methods section. Panels: (*a*) no primary antibody; (*b*) control; (*c*) bGH; (*d*) insulin.

Administration of bGH at 4.5 nM significantly increased ($P < 0.05$) glucose uptake 3.5-fold over control. Insulin significantly increased ($P < 0.05$) glucose uptake to 3.2- and 4.4-fold over control when provided at 10 nM and 100 nM respectively. Co-treatment of adipocytes with both GH and insulin at 4.5 nM and 100 nM respectively did not increase ($P > 0.05$) glucose uptake by comparison with that observed with either hormone at the same level alone.

Stimulation of glucose uptake by insulin occurs rapidly [25]. The results presented in Table 1 suggest that the effect of bGH on glucose uptake may also be rapid, since a 10 min exposure period was sufficient to increase glucose uptake. To investigate further the temporal nature of that interaction, a series of experiments was conducted to determine the effect of treating adipocytes with bGH (4.5 nM) or insulin (100 nM) for 5 min to 4 h before measurement of glucose uptake (Fig. 3). Incubation of adipocytes with either bGH or insulin for 5 min increased ($P < 0.05$) glucose uptake compared with control cells, with longer insulin-treatment periods giving comparable or slightly greater levels of stimulation. The rapid diminution of glucose uptake in cells treated with bGH for longer periods (e.g. 1, 2 or 4 h) contrasts with the less-transient effect of insulin on glucose uptake.

bGH and insulin stimulation of glucose-transporter translocation

To determine whether the mechanism of bGH-stimulated adipocyte glucose uptake is similar to that of insulin (i.e. rapid Glut4 translocation to the plasma membrane), adipocytes were untreated (Fig. 4, both panels, lanes 1 and 2) or treated with 4.5 nM-bGH (lanes 3 and 4) or 100 nM-insulin (lanes 5 and 6) for 20 min and then separated into plasma membrane (lanes 1, 3 and 5) and LDM (lanes 2, 4 and 6). This fractionation procedure effectively separates the internal glucose-transporter pool from plasma-membrane glucose transporters [34,35]. Western-blot analysis of plasma membrane and LDM samples with antisera to Glut4 and Glut1 indicated that bGH and insulin increased plasma-membrane content of Glut4 by 3.5- and 5.0-fold respectively (Fig. 4*a*) and that of Glut1 by 1.5- and 1.9-fold respectively (Fig. 4*b*). bGH and insulin also evoked a concomitant decrease in LDM content of Glut4 and Glut1. Glucose uptake was increased 3.0- and 3.5-fold respectively in parallel unfractionated adipocyte cultures, compared with that in untreated adipocytes.

Immunocytochemical analysis of bGH- and insulin-induced glucose-transporter translocation

Confocal laser microscopic imaging of adipocytes immunocytochemically labelled with anti-Glut4 antisera was used as a second method to determine the effect of bGH and insulin on intracellular Glut4 translocation. Adipocytes were treated for 20 min with 4.5 nM-bGH or 100 nM-insulin, fixed with paraformaldehyde, and incubated first with antisera directed against Glut4 or Glut1 proteins and then with a fluorescein-labelled goat anti-rabbit antibody. No immunofluorescence of cells stained with the fluorescein-labelled antibody alone was observed (Fig. 5*a*). Immunofluorescence in control adipocytes (Fig. 5*b*) labelled with antisera to Glut4 was predominantly localized intracellularly, with little observable labelling of the plasma membrane. However, intense labelling of the plasma membrane was observed, along with cytoplasmic staining in adipocytes treated

with either bGH or insulin (Figs. 5*c* and 5*d* respectively). Because the Glut1 isoform is present in both the plasma membrane and intracellular vesicles in unstimulated adipocytes, it is difficult to detect subtle changes in Glut1 translocation. Consequently, the modest increases in Glut1 translocation induced by bGH or insulin were not readily apparent by the immunocytochemical techniques employed in this study (results not shown).

DISCUSSION

The insulin-like effect of GH on adipocyte glucose uptake has been previously documented in primary adipocyte cultures [17] and cultures of 3T3-F442A adipocytes [18]. The cellular mechanism of action for this effect has not, however, been previously reported. Results from the present study indicate that the rapid translocation of the Glut1 and Glut4 proteins from an intracellular compartment to the plasma membrane is involved in the insulin-like effect of GH.

The study of adipocyte physiology *in vitro* has been facilitated mainly by using cultured primary adipocytes [36] or immortalized cell lines such as 3T3-L1 [37], 3T3-F442A [38] or Ob17 [39]. Among other considerations, the suitability of a particular immortal cell line for specific studies is likely to include the appropriateness of that system as a model of 'normal' adipocytes, balanced against the finite lifespan of a primary adipocyte in culture. The culture system used in the present study, however, combines the use of what appear to be phenotypically normal cells with a culture method that allows extended longevity *in vitro*. Systems such as the present one have typically been employed to study hormonal regulation of preadipocyte differentiation of rodents [40], humans [41] or pigs [42].

The present studies show that primary rat preadipocyte differentiation proceeds rapidly after removal of MID. Accumulation of lipid droplets and expression of GPDH activity are consistent with acquisition of the adipocyte phenotype [30]. Although the source of preadipocytes is white adipose tissue, the differentiated adipocytes are not unilocular. The absence of coalesced lipid droplets may be a consequence of adipocyte growth in a monolayer, as 3T3-L1 adipocytes also exhibit that trait. Interestingly, although it is likely that some preadipocytes may have begun to differentiate *in vivo* and were probably co-isolated with uncommitted preadipocytes, we saw no evidence (i.e. lipid droplets) of continued differentiation in the absence of MID. Spontaneous differentiation of preadipocytes maintained in serum-containing medium for several weeks was not observed.

Robust Glut4 expression was observed during differentiation of rat preadipocytes, consistent with reported changes during 3T3-L1-cell differentiation [34]. The absence of detectable Glut4 protein in non-induced cells suggests that the Glut4 protein is the major glucose-transporter isoform of the newly differentiated adipocytes, as is the case with adipocytes *in vivo* [43]. However, significant expression of Glut1 protein was also observed during differentiation of cells in response to MID treatment, such that the Glut1/Glut4 ratio (4.8) was similar to that previously reported for 3T3-L1 adipocytes [43]. In that study, the Glut1/Glut4 ratio was 4.7 in 3T3-L1 adipocytes on day 9 after initiation of differentiation and varied from 3.0 to 3.8 in adipocytes on days 10–12 after initiation of differentiation [43]. Because the differentiated cells *in vitro* used in the current study begin to detach from the culture dishes 7–8 days after addition of MID, we are unable to determine if the Glut1/Glut4 ratio in our cells also decreases over time. Nevertheless, it is noteworthy that preadipocytes freshly derived from tissue, when differentiated *in vitro*, exhibit a relative glucose-transporter isoform complement similar to that of an immortalized and transformed cell line (3T3-L1). It is possible that differentiation *in vitro* may introduce an aberration in glucose-transporter gene expression, or that the cells are restricted in reaching full terminal differentiation. The presence of contaminating undifferentiated cells may contribute to an overestimation of Glut1 protein level; however, since Glut1 levels also increase in the differentiated cells, it seems likely that errors in estimating relative Glut1 levels are minimal. It would appear that the primary adipocytes differentiated *in vitro* do not resemble primary cultures of adipocytes, in which the Glut4/Glut1 ratio has been estimated to be approx. 12:1 [43].

The stimulation of glucose uptake by bGH in the present culture system is in general agreement with earlier reports that hGH stimulated glucose uptake in primary adipocytes [17] and in 3T3-F442A adipocytes [18]. The broad specificity of hGH for both somatogenic and lactogenic receptors does not preclude the fact that the insulin-like effect of hGH may be mediated via interaction with a lactogenic receptor. However, the present studies suggest that the insulin-like effect of GH is through somatogenic receptors, because (1) adipocyte glucose uptake was not increased by adipocyte stimulation with recombinant bovine prolactin (results not shown), and (2) bGH interacts only with somatogenic receptors.

For GH to exert an insulin-like effect, adipocytes must be cultured in the absence of serum for a period lasting at least several hours. The fact that the insulin-like effects of GH are observed only with adipocytes freshly prepared from hypophysectomized, but not from intact, rats [44] suggests that desensitization of the GH receptor and associated mechanisms may be critical. Adipocytes obtained from intact animals require a period of serum-free incubation to elicit the insulin-like effect. The reason for this treatment is not known, but probably involves the attenuation of GH-induced refractoriness. Recently, it has been suggested that intracellular Ca^{2+} may be involved in the observed refractoriness [45].

The discovery that insulin evokes rapid translocation of glucose transporters from a microsomal fraction to the plasma membrane elucidated a mechanism whereby insulin stimulates glucose uptake in insulin-sensitive cells [19,20]. It has been postulated [17,18], but not proven, that a similar mechanism may be responsible for GH-induced glucose uptake. Data from the present study demonstrate that bGH causes the rapid translocation of Glut4 and Glut1 proteins to the plasma membrane. These observations are consistent with the striking similarity between the acute stimulation of glucose uptake by bGH and by insulin.

The possibility that bGH indirectly affects glucose uptake via IGF-I deserves consideration, since that growth factor has also been shown to stimulate glucose uptake [46]. Because the effect of bGH on glucose-transporter translocation and glucose uptake is extremely rapid (within 5 min), and IGF-I is believed to be transcriptionally regulated by GH, it seems unlikely that a bGH-mediated IGF-I paracrine effect could be elicited within this time frame. In 3T3-L1 adipocytes, immunoreactive IGF-I in the medium is only detectable after at least 12 h exposure to bGH (J. W. Tanner & J. L. Miner, unpublished work). Although we have not determined whether the primary cultures described here synthesize or secrete any IGF-I, if these cells behave in a similar fashion with regard to GH stimulation of IGF-I secretion as do 3T3-L1 cells, then the acute stimulation of adipocyte glucose uptake is probably a direct effect of bGH.

The relatively rapid diminution of the bGH-induced effect contrasts with the persistence of the insulin effect. Whether the decrease in glucose uptake in adipocytes treated with bGH for longer periods (i.e. 1, 2 or 4 h), relative to 15 or 30 min, is a consequence of re-internalization of Glut1 and Glut4 proteins, decreased intrinsic activity, or both remains to be determined. To a lesser extent, insulin-stimulated glucose uptake also wanes after

prolonged treatment, which may be the result of both desensitization and glucose-transporter internalization. We investigated the notion that GH-treated adipocytes might condition the medium either by producing a factor(s) which attenuates the insulin-like effect of GH or by decreasing GH content of the medium via proteolytic degradation or by internalization. Medium from adipocyte cultures which had been exposed to bGH for 4 h increased glucose uptake in adipocyte cultures preincubated for 15 h in serum-free medium (results not shown). Also, the addition of 4.5 nM-bGH to the medium after incubation for 1 h did not restore increased glucose uptake compared with cells receiving no additional bGH, suggesting that depletion of GH from the medium is not involved (results not shown). Our interpretation is that the onset of bGH-mediated anti-insulin effects, for which the cellular mechanisms are currently unknown, is responsible for the decrease in glucose uptake. The attenuation of bGH-stimulated glucose uptake is reminiscent of the homologous densitization effect observed during prolonged β-adrenergic-receptor activation [47].

The existence of two glucose-transporter species in adipocytes (primaries and 3T3-L1 cells) precludes determining precisely which transporter mediates bGH- or insulin-stimulated glucose uptake. Although significant increases in plasma-membrane Glut4 and Glut1 contents occur after treatment of adipocytes with either hormone, the possibility that the intrinsic activity of Glut1 and Glut4 transporters also increases bears consideration. bGH does not stimulate glucose uptake in non-MID-treated cells, which express only the Glut1 protein (results not shown). Consequently, it is tempting to speculate that the effect of bGH is mediated solely via the Glut4 transporter. However, it is unclear whether the requisite cell signalling elements for the GH receptor or for the Glut1 glucose transporter exist in preadipocytes.

Similarities between bGH- and insulin-stimulated glucose uptake suggest the convergence of GH- and insulin-receptor-generated signals at a common point distal to receptor occupancy and proximal to the activation of glucose-transporter translocation and/or Glut4/Glut1 intrinsic activation. Aside from that speculation, little definitive information can be provided as to the exact mechanism(s) which may be involved. It is believed that the tyrosine kinase activity of the insulin receptor mediates the actions of that receptor [48]. It has been proposed that the GH receptor in 3T3-F442A adipocytes also is phosphorylated on tyrosine residues [49,50]. Protein sequence similarity between the cloned GH receptors and other receptors which exhibit tyrosine kinase activity, however, does not support the notion that the GH receptor is a tyrosine kinase. It is possible that the GH receptor may be associated with another protein with tyrosine kinase activity [51].

The mechanism(s) facilitating insulin-induced translocation of glucose transport proteins is not known. Consequently, dissection of the events involved in GH-receptor-mediated translocation cannot be accomplished by using the insulin-receptor signalling pathway as a model. Interestingly, it has been demonstrated that phenylarsine oxide inhibits insulin-stimulated glucose uptake, presumably via interaction with vicinal thiol groups of various proteins [52]. Results from our laboratory show that phenylarsine oxide (35 μM) also inhibits bGH-stimulated glucose uptake (results not shown). The insulin-like effect of GH in adipocytes is not limited, however, to increases in glucose uptake. It has recently been demonstrated that GH, like insulin, increases IGF-II binding to adipocyte membranes from hypophysectomized rats [53].

The physiological relevance of the insulin-like effect of GH is unclear. The requirement that adipocytes be removed from serum, and consequently GH, for a period of several hours to evoke that effect necessitates exposing adipocytes to an environment not likely to be encountered *in vivo*. Although release of GH by the adenohypophysis is pulsatile [54], circulating GH is detectable throughout the secretion pattern. Consequently, adipocytes are probably not normally restricted to exposure to GH at any time. Nevertheless, results from the present study provide a rapid, assayable, end-point of GH-receptor activation. Furthermore, we have identified a family of well-characterized cellular proteins which 'receive' signals transduced from the GH receptor. The determination of cellular processes involved in glucose-transporter translocation, as well as requisite domains of the transporter molecule necessary for such effects, may yield clues as to signalling mechanisms subserving the actions of the GH receptor.

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