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RNA interference-mediated reduction in GLUT1 inhibits serum-induced glucose transport in primary human skeletal muscle cells

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Abstract

Using RNA interference (RNAi), we specifically down-regulate protein expression in differentiated human skeletal myotube cultures. Serum stimulation of myotubes increases glucose uptake. Using a sensitive photolabeling technique, we demonstrate that this increase in glucose uptake is accompanied by increased cell-surface content of glucose transporter (GLUT) 1. Using RNAi, we specifically reduce GLUT1 mRNA and protein expression, leading to inhibition of serum-mediated increase in glucose transport. Thus, we demonstrate the utility of RNAi in a primary human differentiated cell system, and apply this methodology to demonstrate that serum-mediated increase in glucose transport in human skeletal muscle cells is dependent on GLUT1.

Keywords: RNA interference; Human skeletal muscle; Serum stimulation; Glucose transport; GLUT1

Glucose transport across the plasma membrane is mediated by a family of facilitative glucose transporter molecules (GLUTs) [1]. In skeletal muscle, a principal tissue responsible for maintaining whole body glucose homeostasis, GLUT1, and GLUT4 are the primary glucose transporters expressed. In skeletal muscle, insulin-stimulated glucose disposal is mediated via translocation of GLUT4 from an intracellular storage site to the plasma membrane and t-tubule fraction [2,3]. In contrast, immunohistochemical studies reveal that the major abundance of GLUT1 is restricted to the cell surface in skeletal muscle [4,5], with intense labeling corresponding to intramuscular perineural sheaths and endoneural vessels [6]. Thus, in adult skeletal muscle, GLUT1 primarily mediates basal, rather than insulin-mediated glucose uptake [7,8].

A relatively brief exposure to serum (minutes to hours) can induce a substantial increase (~2- to 10-fold) in glucose uptake in cultured cells [9–13]. This phenomenon has been observed using various types of serum (fetal calf, horse, bovine, goat, rabbit, and chicken) in several different cell types including chick embryo fibroblasts, L6 myoblasts, BALB/c 3T3 cells, BHK-21 cells, skeletal muscle myotubes from neonatal rats, and rat vascular smooth muscle cells [9–16]. The mechanisms underlying the serum effects on glucose uptake remain poorly understood, although some studies have demonstrated that de novo protein synthesis is required [14,16]. Serum stimulation of L6 myoblasts was accompanied by increased GLUT1 mRNA with no change in GLUT4 mRNA [13]. Taken together, these results led us to hypothesize that serum-induced increase in glucose uptake of cultured cells is attributable to increased GLUT1.

In contrast to skeletal muscle in vivo, primary human cultured muscle cells contain a relatively greater proportion of GLUT1 compared to GLUT4 [16,17]. Therefore, we applied RNA interference (RNAi) to reduce endogenous GLUT1 expression and thus assess the role of GLUT1 in mediating serum-stimulated glucose transport in primary cultures of human skeletal muscle. RNAi was first described in *Caenorhabditis elegans*, as the phenomena by which double stranded RNA results in sequence-specific gene silencing [18], and in recent

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years, this method has been extended to fungi, plants, drosophila [19,20], and mammalian cell lines [21–24]. Here, we show the utility of RNAi to transfer RNA into primary cultured human cells and mediate specific down regulation of targeted proteins. Additionally, we provide direct evidence that GLUT1 mediates serum-stimulated glucose transport in cultured human skeletal muscle.

Materials and methods

Cell culture. Cell culture media were purchased from Invitrogen, (Invitrogen AB, Stockholm Sweden). Skeletal muscle biopsies were obtained with the informed consent from healthy donors (three male and four female, age 60.4 ± 2.4 years, BMI 24.3 ± 0.8 kg/m^2; plasma glucose concentration 5.3 ± 0.3; and all means $\pm$ SEM, respectively) during scheduled abdominal surgery. The ethical committee at the Karolinska Institute approved all protocols. Satellite cells were isolated, grown, and differentiated as described [25]. Myoblasts from passage II were grown in growth medium (GM) (Hams' F-10 medium containing 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (PeSt)).

Serum was obtained from informed healthy male human donors. Serum was allowed to clot for 30 min at room temperature and separated from whole blood by centrifugation at 3000*g* for 10 min at 4 °C. Serum was aspirated and stored at −70 °C until use.

2-Deoxyglucose uptake. Myotubes were stimulated with serum as specified in figure legends, washed four times with serum-free 1% PeSt–DMEM, and incubated for 4 h with serum free 1% PeSt–DMEM and 5 mM glucose. 2-Deoxyglucose uptake was measured as described [26]. Cell-associated radioactivity was determined by lysing cells with 0.5 N NaOH, followed by liquid scintillation counting. Total cellular protein concentration was measured by the Bradford method (Bio-Rad, Richmond, CA).

siRNA design. Two constructs of siRNA oligos (Table 1) for GLUT1 were designed with 3′ overhanging thymidine dimers, following described procedures [23]. Primers were purchased from Ambion (Ambion, Austin, TX). Target sequences were aligned to the human genome database in a BLAST search to eliminate sequences with significant homology to other genes. Sense and antisense RNAs were annealed following the manufacturer's recommended procedures.

siRNA transfection. Myoblasts were seeded in six well plates at 2–3×10^4 cells/cm^2. At 70–80% confluence, myoblasts were rinsed with sterile PBS and trypsinized with 300 μl 1× trypsin–EDTA for 4 min at 37 °C and trypsin activity was terminated by adding 2 ml/well growth medium (GM) with 1% PeSt. Myoblasts were incubated overnight in a humidified atmosphere of air and 5% CO_2 at 37 °C. At the second day, individual siRNAs (1 μg/ml) were mixed in serum/antibiotic-free DMEM (50 μl) for 5 min and 1 μl of the transfection agent, Lipofectamine 2000 (Invitrogen, Sweden) was mixed and incubated with 49 μl DMEM in a separate tube for 5 min. The two mixtures were combined and mixed gently with agitation at room temperature for 30 min. Myoblasts were washed with sterile PBS twice and 1 ml of serum/antibiotic-free DMEM was added to each well and incubated at 37 °C. siRNA transfection complexes (100 μl) were added to each well and incubated for >16 h. Myoblasts were washed with sterile PBS and 2 ml/well GM was added. Cell death was estimated to be <20%. Cultures were grown for two more days before initiating differentiation as described [27]. Myotubes were deprived of serum for 16 h before serum stimulation. Control cultures were similarly prepared, but without addition of siRNA. No further cell death in cultures exposed to RNAi/Lipofectamine 2000 compared to Lipofectamine 2000 alone.

Analysis of mRNA expression of GLUT4, GLUT1, and β2-microglobulin. Cells were harvested directly for RNA extraction (RNAeasy mini kit, Qiagen, Crawley, UK). RNA extractions were DNase treated before 1 μg mRNA (per 20 μl cDNA) was reverse transcribed (Reverse transcription system, Promega, Southampton, UK). Three microliters cDNA (corresponding to 0.15 μg of total RNA) was amplified with 1× Taqman buffer, 5 mM $MgCl_2$, 200 μM of each dNTP, 200 μM of each primer, 1.25 pM of probe, 0.25 U Amp-Erase Uracil *N*-glycosylase, and 1.25 U AmpliTaq Gold (PE Biosystems, Foster City, CA, USA) in real-time quantitative polymerase chain reaction (RTQ-PCR), using an ABI PRISM 7700 (PE Applied Biosystems, Foster City, CA, USA). The nucleotide sequences are reported in Table 2. cDNA specificity of each primer pair was verified by RT-PCR using both genomic DNA and cDNA as a template. For normalization of RNA loading, control samples were run using β2-microglobulin housekeeping gene. Expression levels were quantified by generating a six-point serial standard curve.

Western blot analysis. Cells were harvested and processed as described [28]. Protein concentration was determined and proteins were separated by SDS–PAGE as described [29]. Membranes were immunoblotted with anti-C-terminal peptide GLUT1 anti-sera as described [30]. Results were quantified by densitometry.

Photolabeling of cell surface GLUT1. Myotubes were incubated at 18 °C for 5 min, rinsed and incubated for 8 min with Krebs–Henseleit bicarbonate buffer (KHB) supplemented with 5 mM Hepes and 0.1% BSA, with 100 μM Bio-LC-ATB-BGPA(4,4′-*O*-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,-trifluoroethyl)benzoyl] amino-1,3-propanediyl bis-D-glucose (a kind gift from Dr. Geoffrey Holman, University of Bath, UK)) [31]. Dishes were then UV irradiated for 3 min. Cells were washed with PBS, solubilized and scraped into 1 ml PBS with 2% thesit ($C_{12}E_9$) and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml antipain, 10 μg/ml leupeptin, and 200 μM PMSF). Solubilized cell extracts were then transferred to microtubes (Sarstedt, Nümbrecht, Germany) and rotated for 60 min at 4 °C. Cell lysates were centrifuged for 10 min at 20,000*g*. The supernatant was collected protein concentration was determined. Equal amount of protein was mixed with 50 μl PBS washed streptavidin–agarose beads (50% slurry, Pierce). The streptavidin–biotin complex was incubated overnight at 4 °C with end to end rotation. Beads were washed three times with PBS–1% thesit, three times with PBS–0.1% thesit, and twice with PBS. Photolabeled glucose transporters were eluted from the beads by boiling (10 min). Samples were directly applied to SDS–PAGE and subjected to electrophoresis.

Results

Time-course and dose–response of serum-induced glucose transport

Cultures were grown to >80% confluence and then induced to differentiate. Cells were then stimulated with

Table 1
GLUT1 siRNA sequence for the two constructs

Name	Sense sequence	Anti-sense sequence
hGLUT1 set A	5′-CACUGGAGUCAUCAAUGCCtt-3′	5′-GGCAUUGAUGACUCCAGUGtt-3′
hGLUT1 set B	5′-UGCUGAUGAUGAACCUGCUtt-3′	5′-AGCAGGUUCAUCAUCAGCAtt-3′

h = human.

Table 2
Primers and probes for real-time quantitative PCR

Gene	Forward primer	Reverse primer	Probe
β2-microglobulin	5′-GCCTGCCGTGTGAACCAT-3′	5′-TTACATGTCTCGATCCCACTTACCTATC-3′	5′-FAM-TGACTTTGTCACAGCCCA-TAMRA-3′
GLUT1	5′-CCTGTGGGAGCCTGCAAA-3′	5′-TCTATACACAGGGCAGGAGTCT-3′	5′-FAM-CACTGCTCAAGAAGAC-TAMRA-3′
GLUT4[a]	5′-GCTACCTCTACATCATCCAGAATCTC-3′	5′-CCAGAAACATCGGCCCA-3	5′-FAM-CTGCCAGAAAGAGTCTGAAG GCCT-TAMRA-3′

[a] The primers and probe sequences have been described in [39].

human serum (100%) for 15, 30, or 60 min. Thereafter, cells were washed and serum-free media were added for a further 5 h, prior to measurement of glucose uptake. A 1 h pre-incubation with serum led to a ~2-fold increase in glucose transport in skeletal muscle cultures (Fig. 1A). A profound stimulatory effect on glucose transport was evident with 10% serum stimulation (Fig. 1B). For subsequent experiments, cells were stimulated with 50% serum for 60 min.

Cell surface abundance of GLUT1 in response to serum stimulation

To determine whether the increased glucose transport in primary human muscle cultures in response to serum stimulation was associated with increased cell surface content of GLUT1, we utilized a sensitive photolabeling technique. Serum stimulation led to a 2.5- to 3-fold increase in GLUT1 appearance at the cell surface (Fig. 2A), with no effect on total GLUT1 protein expression. Thus, increased GLUT1 appearance at the cell surface appears to be the primary mechanism mediating the serum-induced increase in glucose uptake in human muscle cells.

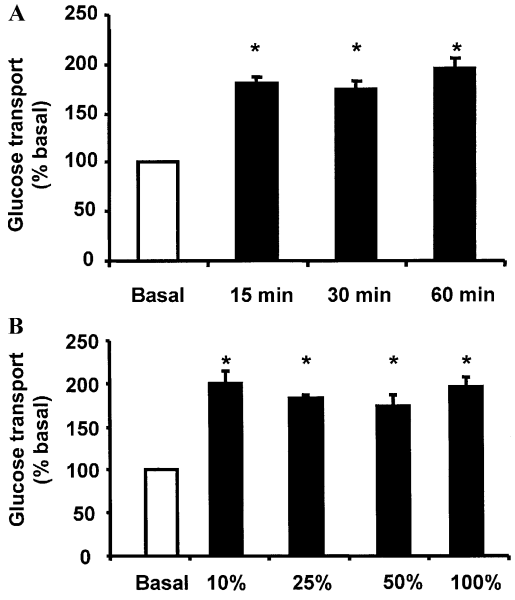

Fig. 1. (A) Time-course and (B) dose–response of serum-induced glucose transport. Differentiated cultures of primary cultures of human skeletal muscles were exposed to (A) 100% human serum for indicated times or (B) Serum at the indicated concentrations for 1 h, after which glucose transport was assessed. Glucose transport is expressed as percent of transport observed in basal (un-stimulated) condition. Figure shows a summary of results (means ± SEM) obtained on cultures derived from four different donors. *, $p < 0.05$ compared to basal.

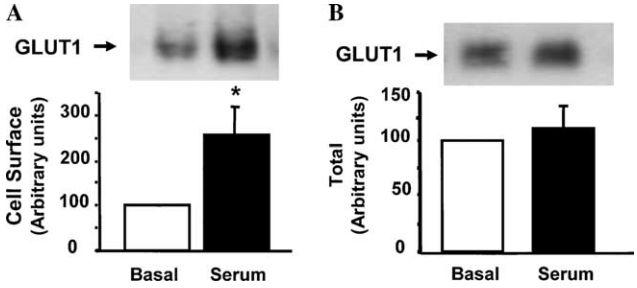

Fig. 2. (A) Cell surface abundance and (B) total expression of GLUT1 in response to serum stimulation. (A) Differentiated cultures of primary cultures of human skeletal muscles were exposed to 50% human serum and the GLUT1 content at the plasma membrane was assessed by photolabeling as described under "Materials and methods." (B) Cultures were incubated as described in A and total protein was extracted for GLUT1 expression analysis by Western blot. Upper panels show a representative Western blot and graph shows summary of results (means ± SEM) obtained on cultures derived from four different donors. *, $p < 0.05$ compared to basal.

Effect of RNAi on protein and mRNA expression of GLUT1 and GLUT4

In order to more specifically address the role of GLUT1 in mediating serum-induced glucose uptake, we utilized RNAi to directly reduce GLUT1 expression in cultured cells. Six days after transfection, protein expression of GLUT1 was measured. Two different RNAi oligo-sequences designed against different portions of the GLUT1 mRNA, (RNAi oligo A and oligo B) were used. The two different RNAi sequences led to ~70% and 90% reduction in GLUT1 protein expression, respectively (Fig. 3A). Muscle cells express GLUT1 and

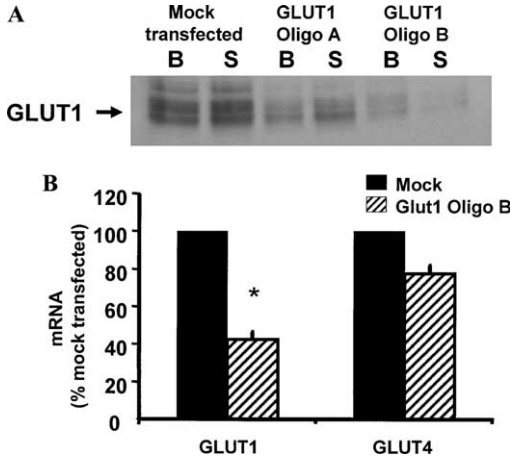

Fig. 3. RNAi mediated reduction in (A) GLUT1 protein expression and (B) mRNA expression. Differentiated cultures of primary cultures of human skeletal muscles were mock transfected or transfected with different RNAi oligos as described under "Materials and methods." Cultures were analyzed for (A) GLUT1 protein expression in total cell lysate by Western blot ($n = 3$ for representative blot) or (B) mRNA of GLUT1 and GLUT4. Solid bar shows mRNA expression in mock transfected cells and hatched bar mRNA expression in RNAi (Glut1 oligoB) transfected cells.

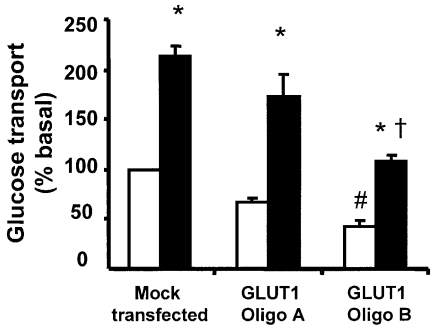

Fig. 4. RNAi mediated reduction in GLUT1 protein expression is accompanied by reduced serum-stimulated glucose uptake. Cells were grown and transfected as described under "Materials and methods." Glucose transport is expressed as percent of basal (un-stimulated) condition. Empty bars are un-stimulated cells and solid bars are serum exposed cells. Graph is means ± SEM on results from cultures derived from three different donors. *, $p < 0.05$ compared to individual basal values; #, $p < 0.05$ compared to mock basal; and †, $p < 0.05$ compared to mock serum.

GLUT4 glucose transporters, encoded by genes with high homology. Thus, we assessed the effect of RNAi-mediated GLUT1 reduction on GLUT1 and GLUT4 mRNA content. As expected, GLUT1 RNAi reduced mRNA content of GLUT1 (Fig. 3B), but was without effect on GLUT4 mRNA content. We have previously shown that insulin-mediated glucose uptake in primary human skeletal muscle cells is mediated via cell-surface appearance of GLUT4 at the plasma membrane, with no effect on GLUT1 [25]. RNAi-mediated reduction in GLUT1 expression did not affect insulin-stimulation of GLUT4 plasma-membrane abundance. (Insulin stimulation was 1.5-fold in both mock and RNAi treated cells, data not shown.)

RNAi-mediated reduction in GLUT1 inhibits serum-induced glucose transport

To determine whether the RNAi-mediated reduction in GLUT1 expression would alter glucose transport, cultures were transfected with RNAi oligoA, or oligoB, and six days after transfection, serum-stimulated glucose transport was assessed (Fig. 4). RNAi-mediated reduction in GLUT1 protein was accompanied by reduced serum-mediated glucose transport. Furthermore, the reduction in glucose transport mirrored the reduction observed in GLUT1 protein expression (Fig. 3A).

Discussion

Using a sensitive photolabeling technique, we demonstrate a serum-dependent appearance of GLUT1 at the cell surface. Furthermore, we have applied the technique of RNA interference to directly show that the serum effect on glucose uptake in primary human muscle

cells is mediated by GLUT1. Taken together, this provides evidence that GLUT1 is a key component of serum-induced increase in glucose transport observed in cultured adult human skeletal muscle. Additionally, we show that RNAi can be directly applied to mammalian tissue to elucidate mechanisms involved in GLUT trafficking. To our knowledge this is the first demonstration of the utility of RNAi to reduce expression of endogenous proteins in primary skeletal muscle cells of human origin.

Classically, transfection of primary cultures of skeletal muscle has been a challenge. Level of transfection in skeletal muscle is inefficient and the preferred methodology has been gene delivery by adenoviral infection [32–35]. Here, we show that some of the challenges with low rates of transfection and high cell death may be circumvented by utilizing RNAi. RNAi has several advantages over other transfection approaches. First, cells are transfected at the single-cellular myoblast stage, when lipophilic agents such as Lipofectamine appear less toxic to cells. Second, the effects of the RNAi on gene expression persist for several days in cultures [23]. As the myoblast cells fuse to form multinucleated myotubes, cells that were not transfected with RNAi are likely to fuse with the transfected cells.

The molecular nature of the serum-mediated increase in glucose transport in skeletal muscle has not been defined. We determined the effect of a serum pre-exposure (1 h), followed by a 5 h incubation in the absence of serum, before assessing glucose transport. Previous reports have shown that serum has a relatively rapid 10–120 min [10,12,14,15] effect on glucose uptake, which may be followed by a plateau and then a further increase after several more hours of serum exposure [10,12,14,15]. The rapid increase in glucose uptake after only 10–15 min cannot be attributed to increased protein synthesis, but with longer serum-treatment ($>$1–5 h), including cycloheximide which during the serum stimulation partially or completely inhibits the serum effect [14,16], suggesting that de novo protein synthesis is required. We postulated that in human muscle cells, serum-mediated increase in glucose uptake could be a reflection of increased GLUT1 protein, as suggested from studies in L6 myoblasts, where a serum stimulation led to an $\sim$300% increase in GLUT1 with no change in GLUT4 mRNA [13]. However, in the current study, serum-pre-exposure was not associated with increased GLUT1 protein content. Instead, our results suggest that in differentiated primary human myotubes, serum-mediated glucose uptake is dependent on GLUT1 redistribution to the cell surface. Furthermore, by applying RNAi and thereby mediating a specific reduction of GLUT1 protein content, we demonstrate that this reduction in GLUT1 inhibits serum-stimulated glucose transport. Thus, GLUT1 expression is necessary for serum effects on glucose uptake.

In summary, pre-exposure of primary differentiated human skeletal muscle myotubes to human serum promotes glucose uptake, mediated by increased GLUT1 content at the cell surface. Furthermore, selective gene regulation using RNAi holds great promise for increasing our understanding of gene and protein function (reviewed in [36]). Application of RNAi to primary human tissues to directly suppress endogenous protein expression, as described in this paper, as well as two recent reports of successful RNAi-mediated gene regulation in primary cultures of rat neurons [37,38] further expands the range of tools that may be applied to mammalian tissue to resolve complicated metabolic interactions.

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