In vitro analysis of the glucose-transport system in GLUT4-null skeletal muscle

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We have characterized the glucose-transport system in soleus muscle from female GLUT4-null mice to determine whether GLUT1, 3 or 5 account for insulin-stimulated glucose-transport activity. Insulin increased 2-deoxyglucose uptake 2.8- and 2.1-fold in soleus muscle from wild-type and GLUT4-null mice, respectively. Cytochalasin B, an inhibitor of GLUT1- and GLUT4-mediated glucose transport, inhibited insulin-stimulated 2-deoxyglucose uptake by $> 95\%$ in wild-type and GLUT4-null soleus muscle. Addition of 35 mM fructose to the incubation media was without effect on insulin-stimulated 3-*O*-methylglucose transport activity in soleus muscle from either genotype, whereas 35 mM glucose inhibited insulin-stimulated (20 nM) 3-*O*-methylglucose transport by 65% in wild-type and 99% in GLUT4-null mice. We utilized the 2-*N*-4-1-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannose-4-yloxy)-2-propylamine (ATB-BMPA) exofacial photolabel to determine if increased cell-surface GLUT1 or GLUT4 content accounted for insulin-stimulated glucose transport in GLUT4-null muscle. In wild-type soleus muscle, cell-surface GLUT4 content was increased by 2.8-fold under insulin-stimulated conditions and this increase corresponded to the increase in 2-deoxyglucose uptake. No detectable cell-surface GLUT4 was observed in soleus muscle from female GLUT4-null mice under either basal or insulin-stimulated conditions. Basal cell-surface GLUT1 content was similar between wild-type and GLUT4-null mice, with no further increase noted in either genotype with insulin exposure. Neither GLUT3 nor GLUT5 appeared to account for insulin-stimulated glucose-transport activity in wild-type or GLUT4-null muscle. In conclusion, insulin-stimulated glucose-transport activity in female GLUT4-null soleus muscle is mediated by a facilitative transport process that is glucose- and cytochalasin B-inhibitable, but which is not labelled strongly by ATB-BMPA.

Key words: GLUT1, GLUT3, GLUT4 knock-out, GLUT5, insulin.

INTRODUCTION

GLUT4 is the primary glucose transporter expressed in skeletal muscle [1–5], a tissue that accounts for the vast amount of whole-body glucose disposal under insulin-stimulated conditions [6,7]. Insulin stimulates glucose uptake in skeletal muscle and adipose tissue primarily by eliciting a translocation of GLUT4 from an intracellular pool to the plasma membrane [4,8–10]. Previously, mice have been generated with the murine *GLUT4* gene disrupted (GLUT4-null) [11]. Surprisingly, GLUT4-null mice display nearly normal glycaemia, and respond to an oral glucose-tolerance test almost as efficiently as wild-type mice [11]. However, GLUT4-null mice demonstrate whole-body insulin resistance, as is evident by both hyperinsulinaemia in the post-prandial state and impaired insulin tolerance [11]. Furthermore, insulin is without effect on glucose uptake in extensor digitorum longus muscles (primarily glycolytic fibres) from both male and female GLUT4-null mice, and soleus muscles (primarily oxidative fibres) from male GLUT4-null mice [12]. Interestingly, in soleus muscles from female GLUT4-null mice, insulin increased glucose-transport activity 2-fold, despite the complete lack of GLUT4 [12]. Thus, a novel compensatory glucose-transport system may participate in glucose transport in female GLUT4-null soleus muscle and reduce the dependence on GLUT4.

In addition to GLUT4 [1–5,13,14], GLUT1 [4,5,15] is expressed in rodent and human skeletal muscle. GLUT5 [16–18] and GLUT3 [19] have also been identified in adult and fetal human skeletal muscle, respectively. Thus an up-regulation of any of the known glucose-transporter proteins could conceivably compensate for GLUT4 ablation in skeletal muscle. However, Northern and immunoblot analyses of GLUT4-null skeletal muscle have failed to detect increased GLUT1 expression or ectopic expression of any other known facilitative glucose-transporter isoform [11,12]. Nevertheless, since GLUT1 is known to translocate to the cell surface of adipocytes in response to insulin [8,20–22], it is a possible candidate to account for the insulin-sensitive increase in glucose transport in soleus muscle from female GLUT4-null mice. The aims of this study were to characterize the glucose-transport system in soleus muscle from female GLUT4-null mice and to determine whether GLUT1, 3 or 5 account for insulin-stimulated glucose-transport activity.

We show first that basal and insulin-stimulated glucose uptake in soleus muscle from female GLUT4-null mice is completely inhibited by cytochalasin B. Additionally, we show that insulin-

Abbreviations used: ATB-BMPA, 2-*N*-4-1-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannose-4-yloxy)-2-propylamine; biotinylated ATB-BMPA, 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-mannose; KHB, Krebs–Henseleit bicarbonate buffer; AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; TBS-T, Tris-buffered saline/0.1% Tween; HES buffer, Hepes/EDTA buffer.

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stimulated glucose transport in GLUT4-null mice is not inhibited by fructose, suggesting that a fructose transporter does not compensate for GLUT4 ablation. Neither GLUT3 nor GLUT5 appear to account for insulin-stimulated glucose-transport activity in soleus muscle from GLUT4-null mice. We determined the affinity for the 2-*N*-4-1-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannose-4-yloxy)-2-propylamine (ATB-BMPA) compound in GLUT4-null soleus muscle by using it as a competitive inhibitor of insulin-stimulated 2-deoxyglucose uptake. Using the ATB-BMPA exofacial photolabel, we show that insulin-stimulated glucose-transport activity in soleus muscle from GLUT4-null mice is not associated with increased cell-surface GLUT1 content. Collectively, these data give additional support for the existence of a novel and unusual insulin-sensitive glucose-transport system in soleus muscle from female GLUT4-null mice.

EXPERIMENTAL

Animals

GLUT4-null mice were obtained and genotyped as described previously by Katz et al. [11]. Female GLUT4-null mice and C57Bl6/CBA F1 hybrids (wild-type) were housed in the animal facility at the Karolinska Hospital and were maintained on a 12-h light/dark cycle. The Animal Ethical Committee of the Karolinska Institute approved all protocols. Animals were given a standard diet of rodent chow and water *ad libitum*. Fed mice (12–16 weeks old) were anaesthetized via intraperitoneal injection of 2.5% avertin (0.02 ml/g of body weight) and soleus muscles were removed for incubation *in vitro*.

Muscle pre-incubations

All incubation media were prepared from a pre-gassed (95% O_2/5% CO_2) stock of Krebs–Henseleit bicarbonate buffer (KHB) [23] supplemented with 5 mM Hepes and 0.1% radioimmunoassay-grade BSA. Pre-incubation media contained 2 mM pyruvate and 18 mM mannitol. Muscles were incubated in 1 ml of medium in a shaking water bath (30 °C). The gas phase in the vial was maintained at 95% O_2/5% CO_2. Muscles were pre-incubated in the presence or absence of insulin or cytochalasin B as described in the Table and Figure legends. When present, insulin and cytochalasin B concentrations were maintained throughout the experiment.

Assessment of glucose-transport activity

In experiments where 2-deoxyglucose was used, muscles were pre-incubated for 40 min in KHB as described above, then transferred to incubation vials containing 0.1 mM [^{3}H]2-deoxyglucose (2.5 μCi/ml) and 19.9 mM [^{14}C]mannitol (0.7 μCi/ml). 2-Deoxyglucose uptake was assessed for 20 min as described by Hansen et al. [24]. When 3-*O*-methylglucose was used, muscles were pre-incubated for 30 min, and thereafter transferred to vials containing 2–40 mM [^{3}H]3-*O*-methylglucose (5.0 μCi/ml) and 0–38 mM [^{14}C]mannitol (0.7 μCi/ml) as described in the Table and Figure legends. In some experiments, 35 mM glucose or fructose was added to the final incubation media, and unlabelled mannitol was used to maintain the osmolarity of KHB supplements at 40 mM throughout the 3-*O*-methylglucose experiments. Muscles were incubated for 15 min (basal) or 7 min (insulin) for the analysis of 3-*O*-methylglucose transport. Muscles were processed as described previously for 2-deoxyglucose uptake [24] and 3-*O*-methylglucose transport [25]. Sample aliquots were used for protein determination using a commercially available kit based on the Bradford method (Pierce, Rockford, IL, U.S.A.). Uptake of glucose analogues is expressed as nmol/mg of protein per 20 min (2-deoxyglucose) or 7 min (3-*O*-methylglucose).

Photolabelling of cell-surface glucose transporters

Following a 30-min pre-incubation, muscles were transferred to a dark room and incubated for 8 min at 18 °C in media containing 1 mCi/ml ATB-[2-^{3}H]BMPA. Muscles were irradiated with UV light for 2 × 3 min, as described by Lund et al. [26]. Thereafter, muscles were blotted, trimmed free of connective tissue and frozen in liquid nitrogen. Photolabelled glucose transporters were immunoprecipitated from each solubilized crude membrane preparation [26] using anti-C-terminal peptide GLUT1 [8] or GLUT4 [8] anti-sera. Thereafter, proteins were separated by SDS/PAGE. Gels were cut into slices and processed as described previously [26,27] for the determination of cell-surface glucose transporters. Results are expressed as c.p.m./100 mg of wet-weight muscle.

Total cell-surface ATB-BPMA labelling was performed using biotinylated ATB-BMPA {4,4′-*O*-[2-[2-[2-[2-[2-[6-(biotinyl-amino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,-trifluoroethyl)benzoyl]amino-1,3-propanediyl bis-D-mannose}. The utility of the compound has been described previously [28]. Soleus muscles from wild-type or GLUT4-null mice were pre-incubated as described above. Muscles were transferred to KHB containing 20 nM insulin, with either 100 mM mannitol or 100 mM glucose, and incubated for 20 min. Muscles were incubated in identical media with the addition of 400 μM biotinylated ATB-BMPA [28], which was activated by UV irradiation. Approx. six soleus muscles (total 30 mg, wet weight) per condition were homogenized in 400 μl of HES (Hepes/EDTA) buffer [255 mM Sucrose, 1 mM EDTA, 20 mM Hepes, 1 μg/ml each of antipain, aprotinin, pepstatin and leupeptin, and 100 μM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), pH 7.2] for 30 strokes at 0–4 °C on ice. Homogenates were washed once with 200 μl of HES buffer and subjected to centrifugation (227000 ***g*** for 50 min at 4 °C) to obtain a total membrane fraction. The pellet was treated with 1 ml of 0.1 M Na_2CO_3, pH 11.3, for 30 min at 4 °C and subjected to centrifugation (227000 ***g*** for 30 min at 4 °C). The pellet was resuspended in 0.5 ml of PBS with 2% of Thesit ($C_{12}E_9$) and protease inhibitors (antipain, aprotinin, pepstatin and leupeptin, each at a concentration of 1 μg/ml, and 100 μM AEBSF) at pH 7.2. Samples were solubilized for 50 min at 4 °C and were then subjected to centrifugation (20000 ***g*** for 20 min at 4 °C). Biotinylated proteins in the supernatants (420 μg) were precipitated with 50 μl of Streptavidin beads (Pierce). The precipitates were washed four times with 1 ml of PBS buffer containing 1% Thesit with protease inhibitors, four times with 1 ml of PBS containing 0.1% Thesit plus protease inhibitors and once in 1 ml of PBS. The pellet was dissolved in 35 μl of electrophoresis sample buffer [62.5 mM Tris, pH 6.8, 2% SDS (w/v), 10% glycerol and 0.01% Bromophenol Blue (w/v)] and heated to 95 °C for 30 min. The samples were subjected to centrifugation (2300 ***g*** for 1 min) and supernatants were removed. The pellets were washed with 30 μl of electrophoresis sample buffer, heated to 95 °C for 30 min, and subjected to centrifugation (2300 ***g*** for 1 min). Both supernatant fractions were pooled and subjected to SDS/PAGE (10% gel). Proteins were transferred on to nitrocellulose membranes. Membranes were blocked with 5% Marval in Tris-buffered saline (20 mM Tris base/140 mM NaCl, pH 7.6)/0.1% Tween (TBS-T) and washed six times with TBS-T. Membranes were incubated with ExtrAvidin horseradish peroxidase conjugate (Sigma, St. Louis, MO, U.S.A.) in TBS-T

containing 1% BSA (45 min at room temperature) followed by washing (six times in TBS-T). Biotinylated ATB-BMPA labelling was visualized by enhanced chemiluminescence (ECL). Two independent experiments were performed.

Western-blot analysis

For each membrane preparation, four soleus muscles from either wild-type or GLUT4-null mice were minced in 0.6 ml of ice-cold homogenization buffer (20 mM Hepes, 135 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) followed by glass-on-glass homogenization (20 stokes). Samples were subjected to centrifugation (12000 ***g*** for 10 min at 4 °C) to remove insoluble material. Thereafter, supernatants were subjected to centrifugation (110000 ***g*** for 50 min at 4 °C). Membrane pellets were resuspended in 0.2 ml of KCl-free homogenization buffer with 0.5% Triton X-100. Samples were solubilized at 4 °C for 45 min. Membranes (25 μg) were solubilized in Laemmli buffer and subjected to SDS/PAGE (7.5% resolving gel). Proteins were transferred to PVDF membranes and blocked with 5% non-fat milk in TBS-T. Membranes were incubated with polyclonal anti-C-terminal-GLUT3 (Biogenesis, Poole, Dorset, U.K.), -GLUT4 [8] or -GLUT5 [29] primary antibodies overnight at 4 °C. Membranes were washed with TBS-T and incubated with anti-rabbit secondary antibody. Proteins were visualized by ECL and quantified by densitometry.

Statistics

To assess the effects between two treatments within a genotype, a paired t test was used; otherwise, differences were examined with a one-way analysis of variance. Fisher's least-significant-difference *post hoc* analysis was used to identify where significant differences occurred. To examine the effects between genotype (wild-type, GLUT4-null), an unpaired t test was used. Significance was accepted at $P < 0.05$.

RESULTS

Cytochalasin B inhibition of 2-deoxyglucose uptake in soleus muscle from GLUT4-null mice

Soleus muscles from female wild-type or GLUT4-null mice were exposed to cytochalasin B, a fungal metabolite that has been used extensively as a competitive inhibitor of GLUT1- and GLUT4-mediated glucose transport [30–34], to determine whether glucose-transport activity in GLUT4-null muscle is mediated via a glucose transporter that shares similar characteristics to GLUT1 or GLUT4. Soleus muscles from wild-type and GLUT4-null mice were used to assess basal or insulin-stimulated (20 nM) 2-deoxyglucose uptake, with or without a further addition of 50 μM cytochalasin B (Table 1). GLUT4 ablation did not alter basal glucose uptake in soleus muscle of GLUT4-null mice compared with wild-type mice. Insulin (20 nM) induced 2.8- ($P < 0.001$) and 2.1-fold ($P < 0.001$) increases in 2-deoxyglucose uptake in soleus muscle from female wild-type and GLUT4-null mice, respectively (Table 1). These results are consistent with previous investigations using GLUT4-null mice [12,35]. Basal and insulin-stimulated 2-deoxyglucose uptake in soleus muscle from wild-type and GLUT4-null mice was inhibited by approx. 95% ($P < 0.05$) following exposure to 50 μM cytochalasin B (Table 1). Furthermore, exposure of insulin-stimulated soleus muscles from wild-type and GLUT4-null mice to concentrations of cytochalasin B ranging from 0.1 to 50 μM led to a dose-dependent inhibition of glucose uptake (Figure 1), which was similar in magnitude between wild-type and GLUT4-null mice. EC_{50} values for cytochalasin B were estimated to be 0.5 and 0.3 μM for wild-type and GLUT4-null mice, respectively.

Table 1 Cytochalasin B inhibition of 2-deoxyglucose uptake in soleus muscles from female wild-type and GLUT4-null mice

Muscles were pre-incubated for 40 min in the presence or absence of 20 nM insulin, with or without 50 μM cytochalasin B. Thereafter, muscles were incubated in KHB containing 0.1 mM 2-deoxyglucose with identical concentrations of insulin and cytochalasin B. 2-Deoxyglucose uptake was assessed for 20 min. Results are expressed as nmol/mg of protein per 20 min. Values represent means ± S.E.M. for 5–13 muscles. *$P < 0.01$ versus basal, †$P < 0.05$ versus wild-type under identical conditions.

	Wild-type	GLUT4-null
Basal	0.54 ± 0.06	0.46 ± 0.03
Insulin	1.49 ± 0.18*	0.95 ± 0.09*†
Basal + cytochalasin B	0.03 ± 0.01*	0.03 ± 0.01*
Insulin + cytochalasin B	0.04 ± 0.02*	0.02 ± 0.01*

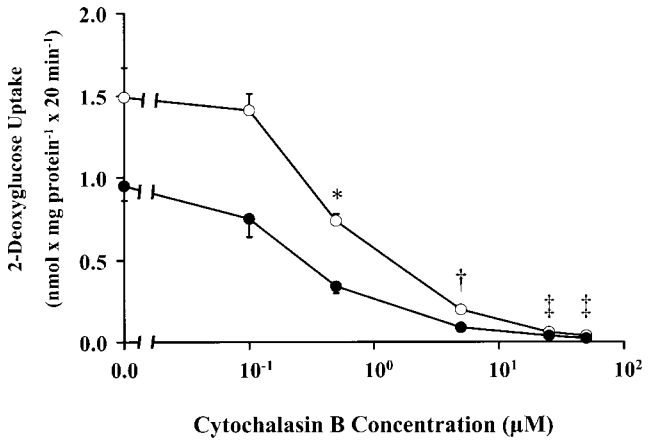

Figure 1 Dose-response inhibition of insulin-stimulated 2-deoxyglucose uptake by cytochalasin B

Soleus muscles from female wild-type (○) or GLUT4-null (●) mice were pre-incubated for 40 min in the presence of 20 nM insulin, with or without cytochalasin B (0.1–50 μM). Thereafter, muscles were incubated in KHB containing 0.1 mM 2-deoxyglucose with identical concentrations of insulin and cytochalasin B. 2-Deoxyglucose uptake was assessed for 20 min. Values represent means ± S.E.M. for 4–13 muscles. *$P < 0.01$ versus 0 and 0.1 μM cytochalasin B for wild-type and GLUT4-null mice, †$P < 0.05$ versus 0.5 μM cytochalasin B for wild-type mice, and ‡$P < 0.01$ and $P < 0.05$ for wild-type and GLUT4-null mice, respectively, versus 0.5 μM cytochalasin B. Wild-type values were significantly greater ($P < 0.05$) than GLUT4-null at all cytochalasin B concentrations except at 50 μM.

Inhibition of insulin-stimulated 3-*O*-methylglucose transport by glucose

Previously, insulin-stimulated 3-*O*-methylglucose-transport activity has been shown to be inhibited by addition of glucose to the incubation media [36]. We implemented a similar strategy in order to test the specificity of the transport system in female GLUT4-null soleus muscle to glucose or fructose, two known transportable sugars. Soleus muscles from female wild-type or GLUT4-null mice were incubated in the absence or presence of 20 nM insulin, in media containing 5 mM 3-*O*-methylglucose, with or without a further addition of 35 mM glucose or fructose. Insulin-stimulated 3-*O*-methylglucose transport was increased

Table 2 Glucose inhibition of insulin-stimulated 3-*O*-methylglucose-transport activity in skeletal muscle from female wild-type and GLUT4-null mice

Muscles were pre-incubated in the presence or absence of 20 nM insulin for 30 min, followed by incubation for 15 min (basal) or 7 min (insulin) in KHB containing 5 mM 3-*O*-methylglucose, with either 35 mM mannitol, fructose or glucose. Results are expressed as nmol/mg of protein per 7 min. Values are means ± S.E.M. for $n = 5$–11 muscles per group. *$P < 0.01$ versus basal and versus insulin + glucose, †$P < 0.001$ versus wild-type mice under identical conditions.

	Wild-type	GLUT4-null
Basal	0.31 ± 0.08	0.24 ± 0.09
Insulin	4.44 ± 0.56*	1.26 ± 0.23*†
Insulin + fructose	4.51 ± 0.51*	1.22 ± 0.24*†
Insulin + glucose	1.54 ± 0.34	0.01 ± 0.01

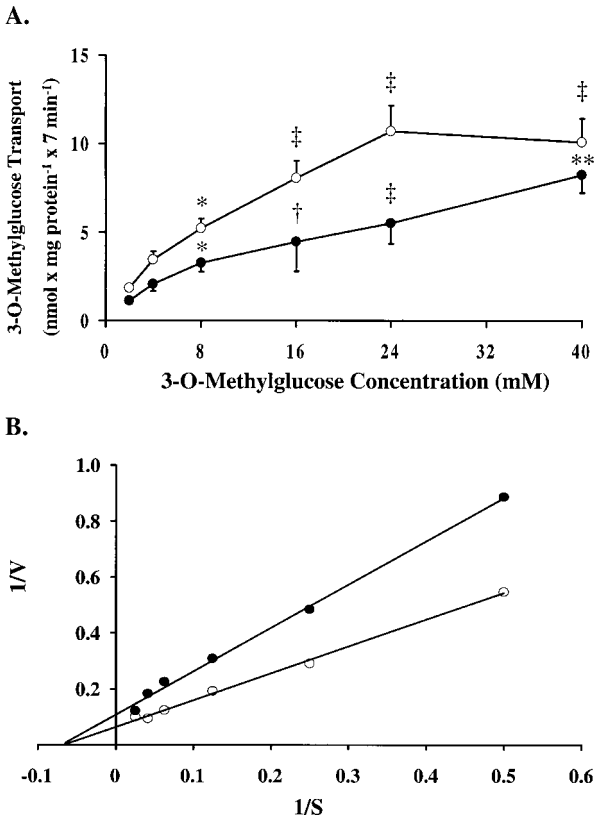

Figure 2 Kinetic analysis of insulin-stimulated 3-*O*-methylglucose transport in soleus muscles from female wild-type (○) and GLUT4-null (●) mice

(**A**) Muscles were pre-incubated for 30 min in the presence of 20 nM insulin. Thereafter, muscles were transferred to vials containing various concentrations of 3-*O*-methylglucose (2–40 mM). 3-*O*-Methylglucose transport was measured for 7 min. Results are means ± S.E.M. for $n = 3$–14 muscles for each group. $P < 0.05$ versus *2, †4, ‡8 and **24 mM glucose. Wild-type values were significantly greater ($P < 0.05$) than GLUT4-null values at 2, 4, 8 and 24 mM glucose. (**B**) Lineweaver–Burk analysis plotting the reciprocals of 3-*O*-methylglucose transport (1/V) versus the reciprocals of 3-*O*-methylglucose concentration (1/S). The K_m values were calculated as 14.3 and 14.7 mM for wild-type and GLUT4-null mice respectively.

14.3- ($P < 0.001$) and 5.3-fold ($P < 0.005$) in soleus muscle from female wild-type and GLUT4-null mice, respectively (Table 2). Addition of fructose to the incubation media did not significantly alter insulin-stimulated 3-*O*-methylglucose transport in soleus muscle from either wild-type or GLUT4-null mice. In contrast, addition of glucose to the incubation media led to a 65% inhibition of insulin-stimulated 3-*O*-methylglucose transport ($P < 0.001$) in soleus muscle from wild-type mice, and completely abolished transport ($P < 0.001$) in soleus muscle from GLUT4-null mice (Table 2).

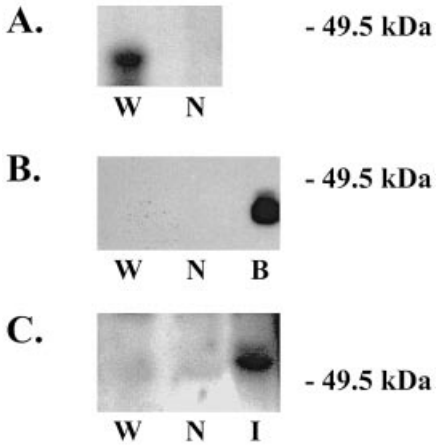

Figure 3 Glucose-transporter protein expression in soleus muscles from female wild-type and GLUT4-null mice

Crude muscle membranes preparations were subjected to SDS/PAGE (7.5% gel) and immunoblotted with polyclonal anti-GLUT4, GLUT3 or GLUT5 antibodies. A representative immunoblot of six membrane preparations for each genotype is shown. (**A**) GLUT4 immunoblot showing the presence or absence of GLUT4 protein in wild-type (W) or GLUT4-null (N) soleus muscle membrane preparations, respectively. (**B**) GLUT3 protein expression in soleus muscle. Crude membranes of brain (B) from wild-type mice were used as positive control. (**C**) GLUT5 protein expression in soleus muscle from wild-type or GLUT4-null mice. Crude jejunal membranes prepared from intestine (I) of wild-type mice were used as a positive control.

Kinetic analysis of 3-*O*-methylglucose transport

Insulin-stimulated glucose-transport activity was assessed in soleus muscles incubated in the presence of increasing concentrations of 3-*O*-methylglucose. In soleus muscles from female wild-type and GLUT4-null mice, a concentration-dependent increase in 3-*O*-methylglucose-transport activity was observed (Figure 2A). Lineweaver–Burk analysis revealed that the calculated K_m for 3-*O*-methylglucose was 14.3 versus 14.7 mM for wild-type versus GLUT4-null mice respectively, with respective calculated V_{max} values equalling 15.5 and 9.2 nmol/mg of protein per 7 min (Figure 2B).

Glucose-transporter expression

As reported previously [12], GLUT4 was detectable in wild-type but not GLUT4-null soleus muscle (Figure 3A), confirming that membrane preparations from GLUT4-null mice were GLUT4-negative. GLUT3 protein was not detected in soleus muscle from either genotype (Figure 3B), whereas a single 50-kDa band was identified in membranes from wild-type brain. A GLUT5-reactive species was detected readily in crude jejunal membranes prepared from intestine of wild-type mice. Over-exposure of the film revealed a GLUT5-immunoreactive protein in the 50-kDa region in soleus muscle membranes from wild-type and GLUT4-null mice (Figure 3C). The expression of this low-abundance GLUT5-reactive species in soleus muscle was similar between wild-type and GLUT4-null mice.

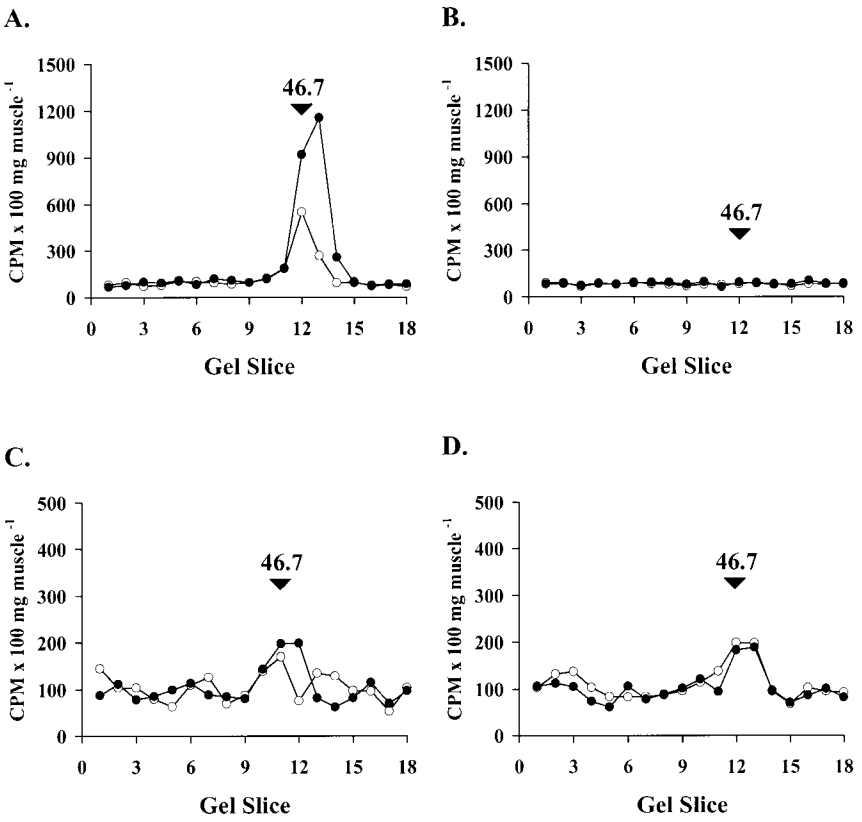

Figure 4 Representative gel profiles of photolabelled GLUT4 and GLUT1 present at the cell surface of basal (○) and insulin-stimulated (●) skeletal muscle

Soleus muscles from female wild-type and GLUT4-null mice were incubated for 30 min in the presence or absence of 20 nM insulin. Thereafter, muscles were processed as described in the Experimental section to assess cell-surface GLUT4 and GLUT1 content. GLUT4 or GLUT1 was immunoprecipitated from solubilized crude membranes with GLUT4 or GLUT1 anti-serum. Immunoprecipitations were separated by SDS/PAGE. Gels were cut into slices and solubilized for determination of cell-surface glucose transporters by scintillation counting of ATB-[2-^{3}H]BMPA. (**A**, **B**) Representative gel profiles for GLUT4 immunoprecipitations in wild-type and GLUT4-null mice, respectively. (**C**, **D**) Representative gel profiles for GLUT1 immunoprecipitations in wild-type and GLUT4-null mice, respectively. Arrowheads indicate gel slices that correspond with the appearance of the 46.7-kDa molecular-mass marker.

Cell-surface glucose transporters in wild-type and GLUT4-null mice

To demonstrate the suitability of the ATB-BMPA compound for use in GLUT4-null soleus muscle, we first incubated muscles in the presence of 20 nM insulin, with or without 500 μM unlabelled ATB-BPMA. Insulin-stimulated 2-deoxyglucose uptake was reduced by 45% when ATB-BMPA was included in the incubation media (1.46 ± 0.10 versus 0.8 ± 0.14 nmol/mg of protein per 20 min without and with ATB-BMPA, respectively; $P < 0.05$). We next assessed basal and insulin-stimulated cell-surface GLUT4 or GLUT1 protein contents using the ATB-[2-^{3}H]BMPA exofacial photolabelling technique. Representative gel profiles are shown in Figure 4. In soleus muscle from female wild-type mice, insulin exposure led to a 2.8-fold increase ($P < 0.005$) in cell-surface GLUT4 content (Table 3). In contrast, essentially no detectable ATB-[2-^{3}H]BMPA labelling was observed in GLUT4 immunoprecipitations from basal or insulin-stimulated soleus muscle from female GLUT4-null mice (Table 3). These results confirm previous reports that GLUT4 protein is completely absent from soleus muscle of GLUT4-null mice [11,12].

We next determined whether insulin-stimulated glucose uptake was mediated by increased cell-surface GLUT1 content. Under basal conditions, cell-surface GLUT1 content was similar between female wild-type and GLUT4-null mice. Furthermore, no detectable increase in cell-surface GLUT1 was observed in either wild-type or GLUT4-null soleus muscle with insulin exposure (Table 3).

Table 3 Cell-surface GLUT4 and GLUT1 contents in soleus muscles from female wild-type and GLUT4-null mice

Muscles were incubated in the presence or absence of insulin, as described in Table 2, and cell-surface GLUT4 or GLUT1 was determined as described in the Experimental section. Results are expressed as c.p.m./100 mg of muscle. Values are means ± S.E.M. for $n = 3$ preparations using three muscles per preparation. *$P < 0.005$ versus basal, †$P < 0.005$ versus wild-type under identical conditions.

	Wild-type	GLUT4-null
Cell-surface GLUT4		
Basal	851 ± 179	92 ± 92†
Insulin	2375 ± 197*	93 ± 93†
Cell-surface GLUT1		
Basal	271 ± 122	370 ± 71
Insulin	365 ± 68	345 ± 38

Inhibition of cell-surface ATB-BMPA photolabelling by glucose

We next determined whether a glucose-inhibitable ATB-BMPA species could be detected in soleus muscle from GLUT4-null

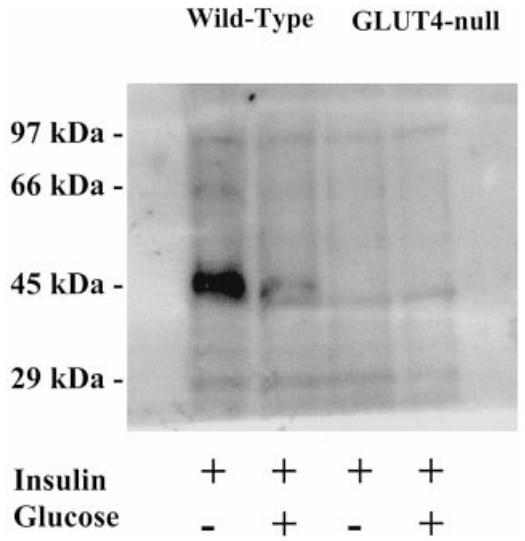

Figure 5 Glucose inhibition of cell-surface ATB-BPMA labelling in skeletal muscle

Soleus muscle from female wild-type or GLUT4-null mice were incubated in 20 nM insulin with (+) or without (−) 100 mM glucose. Mannitol was used to balance osmolarity in the absence of glucose. Thereafter, muscles were incubated in identical media with an addition of 400 μM biotinylated ATB-BPMA. Total membrane proteins prepared from soleus muscles from wild-type or GLUT4-null mice were separated by SDS/PAGE and probed with ExtrAvidin peroxidase as described in the Experimental section. Molecular-mass markers are shown on the left.

mice. This strategy was employed to determine whether a novel glucose transporter(s) is expressed in GLUT4-null mice. Soleus muscles from wild-type or GLUT4-null mice were incubated in 20 nM insulin with or without the addition of 100 mM glucose, and biotinylated ATB-BMPA photolabelling was performed. Samples were visualized with ExtrAvidin peroxidase. Addition of glucose to the incubation media efficiently displaced a predominant ATB-BMPA-labelled band in the 50-kDa region in wild-type soleus muscle preparations (Figure 5). In contrast, no major glucose-inhibitable bands were detectable in GLUT4-null soleus muscle preparations (Figure 5).

DISCUSSION

Glucose transport in skeletal muscle is regulated primarily via GLUT4 [4,5,26,27]. Glucose-transport activity was assessed in soleus muscles in the presence of insulin and increasing concentrations of 3-*O*-methylglucose in order to compare the kinetics of the glucose-transport process in wild-type and GLUT4-null soleus muscle. Lineweaver–Burk analysis of the insulin-stimulated 3-*O*-methylglucose-transport activity revealed similar K_m values between the glucose-transport systems in female wild-type and GLUT4-null soleus muscle. This suggests that, under insulin-stimulated conditions, the affinity properties of the glucose-transport system in soleus muscle from female GLUT4-null mice are comparable with those observed in female wild-type mice, which express GLUT4. However, the V_{max} was lower in soleus muscle from GLUT4-null mice.

Soleus muscles from female wild-type or GLUT4-null mice were exposed to cytochalasin B, a widely used inhibitor of GLUT1- and GLUT4-mediated glucose transport [30–34]. Cytochalasin B inhibited 2-deoxyglucose uptake under basal and insulin-stimulated conditions by up to 98% in soleus muscle from female wild-type and GLUT4-null mice. Thus glucose uptake in soleus muscle from female GLUT4-null mice appears to be mediated by a saturable glucose-transport process, and not merely via increased non-specific plasma-membrane permeability.

In human skeletal muscle, 50 μM cytochalasin B inhibits glucose uptake and metabolism approx. 95%, whereas fructose incorporation to glycogen is not altered [33,34]. Burant et al. [37] have provided evidence that GLUT5-mediated fructose uptake is not inhibited by cytochalasin B and, importantly, that glucose uptake is not mediated by GLUT5. Thus our results showing cytochalasin B inhibition provide evidence against a contribution of GLUT5 to the insulin-stimulated glucose-transport activity observed in soleus muscle from GLUT4-null mice. Nevertheless, we performed immunoblot analysis to determine whether increased GLUT5 expression could account for insulin-stimulated glucose-transport activity in soleus muscle from GLUT4-null mice. GLUT5 protein was detected readily in crude jejunal membranes prepared from mouse intestine. In total membrane preparations of soleus muscle, a GLUT5-immunoreactive protein in the 50-kDa range was detected upon over-exposure of the film, with no apparent difference between wild-type and GLUT4-null mice noted. This suggests that a GLUT5-immunoreactive protein may be a minor contributor to hexose transport in skeletal muscle. Further evidence against the high-affinity fructose transporter GLUT5 as a compensatory mediator of insulin-stimulated glucose transport in GLUT4-null muscle is supported by the failure to detect GLUT5 mRNA by high-stringency Northern-blot analysis [11], as well as by reverse transcriptase-PCR (T. Combatsiaris and M. J. Charron, unpublished work). Additionally, insulin-stimulated 3-*O*-methylglucose-transport activity was not inhibited by the addition of 35 mM fructose to the incubation media. Whereas addition of glucose to the incubation media led to a 65% inhibition of insulin-stimulated 3-*O*-methylglucose-transport activity in soleus muscle from wild-type mice, insulin-stimulated 3-*O*-methylglucose transport was completely inhibited in GLUT4-null soleus muscle.

Other possible candidates that may mediate the insulin-stimulated glucose-transport activity in GLUT4-null muscle include GLUT3 and GLUT1. Although GLUT3 has been identified in fetal human skeletal muscle [19], GLUT3 is does not appear to be expressed in soleus muscle from either adult wild-type or GLUT4-null mice. GLUT1 has been shown to contribute to glucose uptake in skeletal muscle [4,5]. We therefore assessed whether an increase in cell-surface GLUT1 content correlated with the insulin-stimulated glucose-transport activity in GLUT4-null soleus. The 2.1-fold increase in insulin-stimulated glucose-transport activity in female GLUT4-null soleus muscle is unlikely to be accounted for by increased GLUT1 levels (i.e. transporter numbers), as we observe no insulin-stimulated increase in GLUT1 labelling in soleus muscle from GLUT4-null mice. These results are consistent with several previous reports that demonstrate unchanged plasma membrane content of GLUT1 in skeletal muscle following insulin exposure [4,38,39]. However, Lund et al. [5] do report a small increase in GLUT1 labelling in rat soleus muscle. Furthermore, immunohistochemical studies reveal that GLUT1 content is restricted to the cell surface in skeletal muscle [38,40], with intense labelling corresponding to intramuscular perineural sheaths and endoneural vessels [40,41].

In 3T3-L1 adipocytes, catalytic turnover rates of GLUT1 and GLUT4 at the plasma membrane are the same [42]. Here we show that GLUT1 labelling in female wild-type soleus muscle is approx. 1/7 of total cell-surface GLUT4 labelling under insulin-stimulated conditions (Table 3). Assuming that these catalytic turnover rates also apply in muscle, glucose flux via GLUT1 is not likely to be greater than 1/7 of that accounted for by GLUT4

in wild-type soleus muscle. In female GLUT4-null soleus muscle, the rate of 2-deoxyglucose uptake in the insulin-stimulated state is 64 % of that observed in wild-type soleus (Table 1). Therefore, glucose-transport activity is 5-fold greater than can be accounted for by the amount of GLUT1 present at the cell surface, unless a change in GLUT1 catalytic activity has occurred and it has acquired greater catalytic flux per unit of transporter than GLUT4 in the wild-type muscle.

Changes in GLUT1 or GLUT4 catalytic activity have been reported to occur under conditions in which the immunodetectable content in plasma-membrane fractions is unaltered [43,44]. For example, in isolated adipocytes, insulin-stimulated glucose transport is decreased by the addition of isoproterenol [43,44], despite unaltered GLUT4 content in plasma-membrane fractions measured by either cytochalasin B binding [43] or immunoblotting [44]. Similarly, anisomycin and noradrenaline increase glucose transport in white [45] and brown [46] cultured adipocytes, respectively, with no detectable increase in immunoreactive GLUT1 or GLUT4 in the plasma membrane. However under these conditions, ATB-BMPA labelling parallels changes in glucose transport despite unaltered immunoreactive GLUT1 [45,46] or GLUT4 [47] content in plasma-membrane fractions. In this regard, it is important to note that, in female GLUT4-null soleus, ATB-BMPA-labelled GLUT1 was unchanged under insulin-stimulated conditions, despite a marked increase in glucose-transport activity. Therefore, if changes in GLUT1 account for the insulin-stimulated increase in glucose-transport activity in the female GLUT4-null soleus, then this occurs unusually as a result of increased flux through a constant number of transporters. Since there are technical difficulties in addressing glucose-transporter kinetics in intact skeletal muscle preparations, the potential role of increased catalytic flux through GLUT1 cannot be completely discounted.

Glucose-inhibitable ATB-BMPA photolabelling was determined in wild-type and GLUT4-null soleus muscle. Addition of 100 mM glucose to the incubation media led to a marked decrease in biotinylated ATB-BMPA photolabelling in wild-type muscle. Presumably, this effect can be largely attributed to competition between glucose and ATB-BPMA for binding to GLUT4. In contrast, no major glucose-inhibitable band(s) were detected in GLUT4-null mice. Therefore, the presence of a compensatory glucose transporter in GLUT4-null muscle is not readily detectable by ATB-BPMA photolabelling when total membrane preparations are analysed.

In conclusion, a facilitative transport process that is glucose- and cytochalasin B-inhibitable, and independent of GLUT 3 or GLUT5, mediates insulin-stimulated glucose-transport activity in soleus muscle from female GLUT4-null mice. Furthermore, increased cell-surface GLUT1 levels do not appear to compensate for GLUT4 ablation. However, the possibility of increased glucose flux through the available GLUT1 cannot be ruled out completely. Whether genetic ablation of GLUT4 promotes the sustained expression or up-regulation of a novel glucose-transport protein that is present at low levels but which has acquired very high catalytic activity to meet the metabolic demands of the developing mouse remains to be determined.

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