Regulation of cell surface GLUT4 in skeletal muscle of transgenic mice

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Marked overexpression of the glucose transporter GLUT4 in skeletal muscle membrane fractions of GLUT4 transgenic (TG) mice is accompanied by disproportionately small increases in basal and insulin-stimulated glucose transport activity. Thus we have assessed cell surface GLUT4 by photolabelling with the membrane-impermeant reagent 2-N-[4-(1-azi-2,2,2-trifluoro-ethyl)benzoyl]-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) and measured the corresponding glucose transport activity using 2-deoxyglucose in isolated extensor digitorum longus (EDL) muscles from non-transgenic (NTG) and GLUT4 TG mice in the absence and presence of 13.3 nM (2000 μ-units/ml) insulin, without or with hypoxia as a model of muscle contraction. TG mice displayed elevated rates of glucose transport activity under basal and insulin-stimulated conditions, and in the presence of insulin plus hypoxia, compared with NTG mice. Photoaffinity labelling of cell surface GLUT4 indicated corresponding elevations in plasma membrane GLUT4 in the basal and insulin-stimulated states, and with insulin plus hypoxia, but no difference in cell surface GLUT4 during hypoxia stimulation. Subcellular fractionation of hindlimb muscles confirmed the previously observed 3-fold overexpression of GLUT4 in the TG compared with the NTG mice. These results suggest that: (1) alterations in glucose transport activity which occur with GLUT4 overexpression in EDL muscles are directly related to cell surface GLUT4 content, regardless of the levels observed in the corresponding subcellular membrane fractions, (2) while overexpression of GLUT4 influences both basal and insulin-stimulated glucose transport activity, the response to hypoxia/contraction-stimulated glucose transport is unchanged, and (3) subcellular fractionation provides little insight into the subcellular trafficking of GLUT4, and whatever relationship is demonstrated in EDL muscles from NTG mice is disrupted on GLUT4 overexpression.

INTRODUCTION

Skeletal muscle is the primary tissue responsible for the insulin-stimulated disposal of a glucose load, and is a major site of insulin resistance in non-insulin-dependent diabetes mellitus. Glucose uptake into muscle can be stimulated by two separate pathways; one pathway is activated by insulin and the other by muscle contraction [1–4]. Increases in glucose transport are mediated by the GLUT4 transporter isoform, which appears to be translocated to the plasma membrane from an intracellular storage site in response to both stimuli [2,5]. It has previously been shown that increased expression of GLUT4 in skeletal muscle produces changes (lowered blood glucose, increased insulin- and contraction-stimulated glucose transport) that could be of benefit in the treatment of non-insulin dependent diabetes [6–10].

Recently, several lines of transgenic (TG) mice that overexpress the human GLUT4 (hGLUT4) gene in a tissue-specific (adipose tissue, cardiac and skeletal muscle) manner have been described [8–10]. These animals are hypoglycaemic and hypoinsulinaemic compared with their non-transgenic (NTG) littermates, and show a markedly increased ability to dispose of an intravenous or oral glucose load [9–11]. Additionally, isolated epitrochlearis, extensor digitorum longus (EDL) and soleus muscles from these animals show increased basal and insulin-stimulated glucose

transport [9,10]. Previously, we developed TG mice that overexpress GLUT4 in a tissue-specific fashion on the inbred C57BL/KsJ-$m+/+db$ background [8]. Preliminary characterization of several lines of these TG mice indicated that the phenotype was similar to that of GLUT4 TG mice developed on a hybrid background. However, it was crucial to fully characterize this line of hGLUT4 TG mice to determine whether the lower level of GLUT4 overexpression ($\sim$ 3-fold) on an inbred genetic background resulted in any significant differences in the physiological profile compared with the well characterized hGLUT4B and hGLUT4C lines [10,12], in order to make valid comparisions with earlier studies. In the present study, we used one line (designated line 8 in [8]) of hGLUT4 C57BL/KsJ-$m+/+db$ mice to investigate GLUT4 subcellular localization under basal, insulin-stimulated and hypoxia-stimulated conditions [8].

Previous examinations of the subcellular distribution of GLUT4 in GLUT4 TG mice by immunofluorescence or membrane fractionation have shown a marked increase in basal and insulin-stimulated plasma membrane GLUT4 content [9,10,13]. However, immunofluorescence is not sensitive enough to detect the difference between plasma membrane and sub-plasma membrane GLUT4. Furthermore, the membrane fractionation technique is problematic at best due to difficulties with cross-contamination of membrane pools, low recoveries of membrane markers and the lack of a marker for the intracellular GLUT4

pool. Additionally, immunoblotting of isolated membrane fractions for GLUT4 does not distinguish between 'active' and 'sequestered' GLUT4 transporters [14]. Indeed, a previous study from our laboratory has shown that the increases in basal and insulin-stimulated plasma membrane GLUT4 content in TG mice are not reflected by similar fold increases in glucose transport activity [13]. Therefore it is not known if the increased plasma membrane GLUT4 includes inactive or sequestered GLUT4.

The novel membrane-impermeant glucose transporter photo-affinity label 2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) has been used to quantify cell surface GLUT4 content in adipose cells in the basal and insulin-stimulated states, and in skeletal muscle in the basal, insulin-stimulated and contraction-stimulated states; this method is free of the limitations associated with the immunofluorescence and membrane fractionation procedures [14–16]. The purpose of the present study was to assess the amount of cell-surface-labelled GLUT4 under basal, insulin-stimulated and hypoxic conditions in GLUT4 TG mice, and to determine the relationship between cell surface GLUT4 and glucose transport activity. Furthermore, the present study provides a direct comparison of our recently developed mouse skeletal muscle subcellular fractionation protocol, requiring only small amounts of starting material, with the photolabelling procedure, as an assessment of the validity of the former method.

EXPERIMENTAL

TG mice

TG mice carrying 11.8 kb (NotI fragment) of the hGLUT4 transporter genomic DNA were produced as described previously [8]. One line of male TG mice (hGLUT4db/+ line 8; [8]), 10–12 weeks of age and hemizygous (except as noted) for the hGLUT4 transgene, and lean NTG C57BL/KsJ-m+/+db mice (as controls) were used in this study. hGLUT4db/+ line 8 displays a 3–6-fold overexpression of GLUT4 in cardiac muscle and white adipose tissue [8]. All mice used in this study carried only a single copy of the db gene, and therefore exhibited a lean, non-diabetic, phenotype. The presence of the hGLUT4 transgene was assessed by PCR of total genomic DNA isolated from tail snips, as described previously [12].

Muscle preparation and incubation

Mice in the post-prandial state weighing ~ 30 g were anaesthetized with 5 mg of sodium pentobarbital/100 g body weight. The EDL muscles were dissected out, blotted on gauze and transferred to 25 ml Erlenmeyer flasks containing 2 ml of Krebs–Henseleit buffer (KHB), which contained 0.1% BSA, 32 mM mannitol and 8 mM glucose, with or without 13.3 nM (2000 μ-units/ml) insulin. Hypoxic muscles were placed in similar medium containing KHB that had been previously gassed with 95% N_2/5% CO_2. The flasks were incubated in a shaking water bath maintained at 35 °C for 45 min, and were continuously gassed with 95% O_2/5% CO_2 (normoxic muscles) or 95% N_2/5% CO_2 (hypoxic muscles).

Measurement of glucose transport activity

Glucose transport activity was measured using 2-deoxyglucose (2DG) as described previously [9]. Briefly, after the initial 60 min incubation, muscles were blotted and transferred to flasks containing 2 ml of oxygenated KHB plus 0.1% BSA, 40 mM mannitol, 2 mM pyruvate and insulin at the same concentration that was present during the prior incubation. These flasks were

incubated at 29 °C in a shaking incubator for 10 min to wash out glucose and reoxygenate the hypoxic muscles. For measurement of 2DG uptake, the muscles were blotted and transferred to a flask containing 1.5 ml of KHB with 1 mM 2-deoxy[1, 2-^{3}H]glucose (2.25 mCi/mmol) and 39 mM [1-^{14}C]mannitol (8 μCi/mmol), plus 13.3 nM insulin if present in the previous incubation. The transport flasks contained a gas phase of 95% O_2/5% CO_2, and were incubated at 29 °C in a shaking incubator for 20 min. After the incubations, the muscles were frozen in liquid nitrogen until they were processed for measurement of 2DG transport. Frozen muscles were homogenized in 1 ml of 0.6 M perchloric acid. Homogenates were centrifuged at 5000 g, and aliquots of the supernatant were counted for radioactivity in a liquid scintillation counter. The c.p.m. values were corrected for channel crossover, and transport was calculated as μmol/20 min per ml after correcting for the extracellular space as calculated from the [^{14}C]mannitol counts.

Photoaffinity labelling of isolated EDL muscles

Following the incubation and wash steps described above, isolated EDL muscles were incubated in 1 ml of KHB containing insulin at the same concentration as in the previous incubation and 1 mCi/ml ATB-[^{3}H]BMPA (kindly provided by Dr. G. Holman, University of Bath, Bath, U.K.) for 5 min at 25 °C. Each muscle was then irradiated for 2×2 min intervals in a photometer lamp. The muscles were manually turned over between intervals. Following irradiation, the muscles were trimmed of their tendons, blotted and frozen between liquid-nitrogen-cooled clamps. Muscles were stored at -70 °C until processed.

Photolabelled muscles were solubilized and GLUT4 was immunoprecipitated as described previously [15–17]. Briefly, Thesit (Boehringer Mannheim, Indianapolis, IN, U.S.A.)-solubilized crude membranes were incubated with an immuno-complex consisting of anti-(GLUT4 C-terminus) antiserum and Protein A–Sepharose overnight at 4 °C. Subsequent to immunoprecipitation, the immunocomplex was washed two times in 0.2% Thesit buffer and then three times in PBS. The GLUT4–antibody conjugate was then released from the Protein A–Sepharose with SDS/urea sample buffer. Immunoprecipitated GLUT4 was then subjected to SDS/PAGE on 10% resolving gels. Gels were stained with Coomassie Blue, destained and sliced by lane into 8 mm slices. The slices were dried and solubilized in 30% H_2O_2 and 2% ammonium hydroxide, and the radioactivity was quantified in a liquid scintillation counter. Labelled GLUT4 protein, expressed as d.p.m./mg wet weight, was quantified by integrating the area under the ^{3}H peak corresponding to the 45 kDa molecular mass marker and subtracting the average background radioactivity in the gel.

Muscle stimulation

For the preparation of membrane fractions, mice were left untreated or were treated with insulin plus contraction in vivo to give maximal GLUT4 translocation. TG or NTG mice in the fed state were given glucose (2 g/kg body weight) and insulin (6 units) intraperitoneally 30 min prior to stimulation of muscle contraction. Immediately prior to contraction the mice were anaesthetized with 5 mg of sodium pentobarbital/100 g body weight. Contraction was initiated by electrical stimulation of the sciatic nerves by 200 ms trains of 100 Hz, with each impulse in a train being 0.1 ms. The trains were delivered at a rate of 1 per s at 12 V for 2×5 min separated by a 1 min rest period. After contraction, the hindlimb muscles innervated by the sciatic nerve were removed and frozen in liquid nitrogen. Unstimulated control

mice were anaesthetized, and the same hindlimb muscles were removed and frozen.

Membrane preparation

The plasma membrane and intracellular membranes were prepared as described previously [13]. Briefly, approx. 1.0 g of mixed mouse hindlimb skeletal muscle was removed at the end of stimulation, cleaned of fat and connective tissue, weighed and frozen in liquid nitrogen. This muscle was pulverized in a mortar and pestle under liquid nitrogen. The pulverized muscle was homogenized with a Brinkman PT-10 Polytron (Brinkman Instruments, Inc., Westbury, NY, U.S.A.) at slow speed and then homogenized again with a Potter–Elvehjem tissue grinder, and brought up to a volume of 15 ml with homogenization buffer. A 0.2 ml aliquot was removed for measurement of marker enzymes and protein, and the homogenate was then centrifuged at $34000\,g$ for 20 min. The resulting pellet was used for purification of plasma membranes, while the supernatant was used for the purification of intracellular membranes, as described in detail in [6].

Each intracellular membrane and plasma membrane suspension was weighed to determine the exact volume. An aliquot of each suspension was removed for marker enzyme and protein determinations, and the remainder was used for Western blotting. All samples were stored at $-70\,°C$ until assayed.

Protein and marker enzyme assays

Homogenate and membrane proteins were determined for each preparation by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.) using crystalline BSA as the standard. K^+-stimulated p-nitrophenolphosphatase (KpNPPase) specific activity was measured as a marker for plasma membranes by the method of Bers et al. [18].

Tissue preparation for determination of hexokinase activity and total GLUT4 content

Muscle samples were weighed and then homogenized on ice at high speed with a Brinkman Polytron homogenizer for 2×15 s bursts in a buffer containing 20 mM Hepes, 1 mM EDTA and 250 mM sucrose, pH 7.4, 1:20 (w/v) at 4 °C. For measurement of hexokinase activity, an aliquot of the initial homogenate was further diluted 2:3 in 0.1 M Tris(hydroxymethyl)aminomethane-HCl and 4 % Triton X-100 (pH 8.1), vortexed and centrifuged at $13600\,g$ for 5 min at 25 °C. The supernatant was assayed for hexokinase activity by the method of Uyeda and Racker [19], using glucose as a substrate and monitoring the reduction of $NADP^+$ spectrophotometrically.

Western blotting

Plasma membrane and intracellular membrane protein (30 μg of sample protein) and crude homogenate protein (100 μg of sample protein), along with molecular mass markers (Gibco-BRL, Gaithersburg, MD, U.S.A.), were subjected to SDS/PAGE under reducing conditions using a 12 % resolving gel as described previously [20]. Membrane samples from each group of mice were run in adjacent lanes. Resolved proteins were transferred to poly(vinylidene difluoride) (PVDF) sheets (Bio-Rad, Richmond, CA, U.S.A.) as described previously [21] using a Bio-Rad SD semi-dry transfer unit. After transfer, the PVDF sheets were blocked in 20 mM Tris and 500 mM NaCl (TBS), pH 7.5, containing 5 % non-fat dry milk, at 25 °C. Next, the sheets were washed in TBS containing 0.05 % Tween-20 (TTBS) for 20 min

and then incubated overnight with the polyclonal anti-(GLUT4 C-terminus) antibody (8107P; kindly provided by Hoffman-La Roche, Nutley, NJ, U.S.A.) in TTBS, 3 % BSA and 0.02 % sodium azide. The PVDF was then washed in TTBS for 20 min followed by incubation with ^{125}I-Protein A (New England Nuclear, Boston, MA, U.S.A.) at a concentration of 0.25 mCi/ml for 1 h. The sheets were then washed in TBS and air dried. Antibody binding was visualized by exposure to a Phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA, U.S.A.) for 72 h. Each band was corrected for background and the area of each band was expressed as a percentage of that of a standard (50 μg of rat skeletal muscle intracellular membrane protein) run on each gel.

Oral glucose tolerance test and metabolite measurements

Non-anaesthetized GLUT4 TG and age-matched NTG mice were fasted overnight and bled via the orbital sinus (0.025 ml) immediately prior to administration of an oral glucose load (1 g of glucose/kg body weight) by gavage using a syringe equipped with a murine oral feeding needle (20 gauge; Popper & Sons, New Hyde Park, NY, U.S.A.). Mice were subsequently bled after 30, 75 and 120 min. Plasma glucose and β-hydroxybutyrate was measured using a VP Super System Autoanalyzer (Abbott Laboratories, North Chicago, IL, U.S.A.). The total plasma cholesterol profile was determined using FPLC post column reaction using cholesterol/horseradish peroxidase reagent (Boehringer Mannheim). Plasma insulin and glucagon were determined using radioimmunassays (Linco Research, St. Charles, MO, U.S.A., and ICN Biomedicals, Costa Mesa, CA, U.S.A., respectively). Quadriceps, diaphragm, heart and liver samples were isolated from fed or overnight (18 h)-fasted mice and rapidly frozen in liquid nitrogen; tissue glycogen levels were then determined by the method of Hassid and Abraham [22].

Statistical analysis

The data were analysed by ANOVA to test the effects of group (NTG versus TG) and treatment (basal, insulin, hypoxia and hypoxia plus insulin) on muscle glucose uptake and glucose transporter distribution. When a significant F ratio was obtained, the Newman–Keuls *post hoc* test was employed to identify statistically significant differences ($P < 0.05$) between the means.

RESULTS

Western blot analysis of gastrocnemius muscles from NTG and TG mice was performed to determine the level of overexpression of GLUT4. GLUT4 immunoreactivity in TG mice was approx. 3-fold greater than in NTG mice (9.20 ± 0.99 and 3.05 ± 0.82 relative Phosphoimager units respectively), similar to previous reports [8].

Metabolic data for the NTG and TG mice are given in Tables 1 and 2. Plasma glucose levels in both fed and fasted animals were significantly lower in TG than in NTG mice. However, unlike our initial studies [8] with this line of mice (hGLUT4*db*/+ line 8), in which we failed to detect a difference in fasting plasma glucose levels compared with NTG mice, we now consistently observed a significant decrease in this parameter (Table 1). Similarly, plasma insulin levels were significantly lower in TG than in NTG mice in the fed state, which indicates an increased sensitivity to insulin. Following an overnight fast, plasma insulin concentrations were significantly decreased in both groups of mice, but did not differ between TG and NTG mice. Plasma glucagon levels in fed animals were significantly higher in TG

Table 1   Plasma insulin and glucagon levels and muscle glycogen levels in NTG and TG *db/+* mice in the fed and fasted state

Values are means ± S.E.M. All values for TG mice are from hemizygotes, except for fed glucose levels, which are from homozygotes. For glycogen measurements, $n = 6$ NTG mice, except for fed and fasted quadriceps ($n = 11$ and $n = 8$ respectively) and fasted heart ($n = 8$); $n = 5$ TG mice in all cases. For insulin and glucagon levels, n is given in parentheses. *, Significantly different from NTG mice ($P < 0.05$); †, significantly different from corresponding fed value ($P < 0.05$).

Parameter	Fed		Fasted	
	NTG	TG	NTG	TG
Glucose (mg/dl)	190 ± 11 (7)	160 ± 8 (7)*	77.9 ± 3.9 (16)†	52.9 ± 7.8 (10)*†
Insulin (ng/ml)	72.0 ± 7.5 (19)	48.9 ± 4.8 (12)*	14.8 ± 3.6 (11)†	22.1 ± 3.4 (9)†
Glucagon (pg/ml)	193 ± 14 (11)	262 ± 40 (8)*	284 ± 26 (12)†	245 ± 36 (10)
Tissue glycogen (μmol/g)				
Diaphragm	7.3 ± 1.3	8.4 ± 0.8	3.1 ± 1.0†	3.7 ± 0.4†
Heart	3.8 ± 0.3	16.0 ± 3.9*	3.1 ± 0.4	24.0 ± 3.0*†
Liver	218 ± 27	214 ± 27	16.4 ± 3.8†	26.5 ± 2.4*†
Quadriceps	5.7 ± 2.0	11.3 ± 0.9*	4.7 ± 0.7	5.6 ± 1.4†

Table 2   Plasma cholesterol profiles for NTG and TG *db/+* mice

Values are means ± S.E.M. *, Significantly different from NTG value ($P < 0.05$).

Mice	Cholesterol (mg/dl)			
	VLDL	LDL	HDL	Total
NTG	3.82 ± 0.51	9.26 ± 0.79	84.20 ± 5.8	122 ± 8.0
TG	5.56 ± 0.77	2.53 ± 0.60*	65.4 ± 3.2*	91.8 ± 4.1*
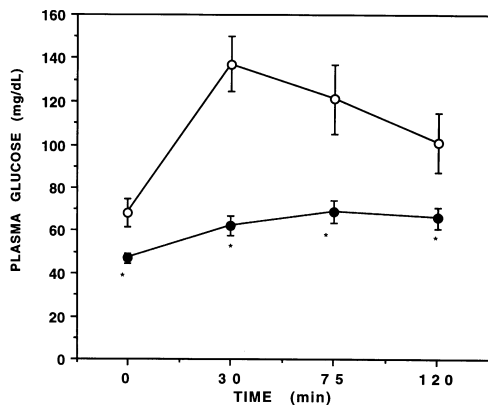
Figure 1   Effect of an oral glucose challenge on plasma glucose levels in TG and NTG *db/+* mice

NTG and homozygous TG mice were fasted overnight and then given 1 g of glucose/kg body wt. by oral gavage. Plasma glucose levels were determined at the times indicated. Values are expressed as means ± S.E.M. from six TG (●) and five NTG (○) mice. *Significantly different from NTG mice ($P < 0.05$).

than in NTG mice. Fasting did not alter plasma glucagon in TG mice, whereas it produced a significant elevation in NTG mice. Total plasma, high-density lipoprotein and low-density lipoprotein cholesterol were significantly reduced in TG compared with NTG mice, whereas very-low-density lipoprotein cholesterol was unchanged (Table 2).

The effects of an oral glucose challenge on plasma glucose levels in overnight-fasted TG and NTG mice are shown in Figure 1. Similar to our previous results [8], 30 min after an oral glucose load (1 g/kg body weight) a substantial transient increase in the plasma glucose level (approx. 2-fold) occurred in NTG mice. In marked contrast, TG mice displayed only a minimal increase in plasma glucose in response to the same glucose load, superimposed on the already lower basal fasting glucose levels at zero time (Figure 1). At the end of the glucose tolerance test ($t = 120$ min), mice were killed and plasma β-hydroxybutyrate levels were determined. Consistent with our earlier results with hGLUT4 TG mice on a hybrid background, β-hydroxybutyrate was significantly elevated in TG compared with NTG mice (51.9 ± 3.8 and 21.3 mg% respectively; $P < 0.05$), suggesting that the TG mice are undergoing enhanced lipolysis and subsequent conversion of non-esterified fatty acids into ketones.

In the fed state, the glycogen concentration was significantly increased in quadriceps and heart (2- and 4-fold respectively) in TG mice compared with NTG mice. A small (1.2-fold), but insignificant, increase was also observed in the diaphragm. An overnight fast led to a decrease in the glycogen content in both skeletal muscles, whereas this was not observed in cardiac muscle. In fact, a significant 1.5-fold increase in the fasted glycogen content in hearts from TG mice occurred following the overnight fast; a similar and more pronounced phenomenon was also reported for a different line of GLUT4 TG mice [23]. Hepatic glycogen content was similar in TG and NTG mice in both the fed and fasted states, with a substantial glycogen depletion ($\sim 90\%$) occurring on fasting in both groups of animals.

2DG transport rates in isolated EDL muscles are presented in Figure 2(B). In the basal state, EDL muscles from TG mice exhibited significantly higher rates of 2DG transport than EDL muscles from NTG mice. Insulin, hypoxia and hypoxia plus insulin significantly increased the rate of 2DG transport above basal in EDL muscles from both NTG and TG mice. However, the rates of 2DG transport in response to insulin and hypoxia plus insulin were significantly higher in TG compared with NTG mice.

Because we found a decrement in gastrocnemius hexokinase activity in TG compared with NTG mice in the present study (12.02 ± 0.53 and 15.07 ± 0.75 μmol/min per mg of protein respectively; $P < 0.05$), we also measured 2DG transport using 0.1 mM 2DG, to ensure that our 2DG transport measurements were not compromised by a limitation in the phosphorylation of glucose [9]. The patterns for the rates of 2DG transport when measured at 0.1 mM 2DG (results not shown) were identical with those when transport was measured at 1.0 mM 2DG.

Panel A

Panel B
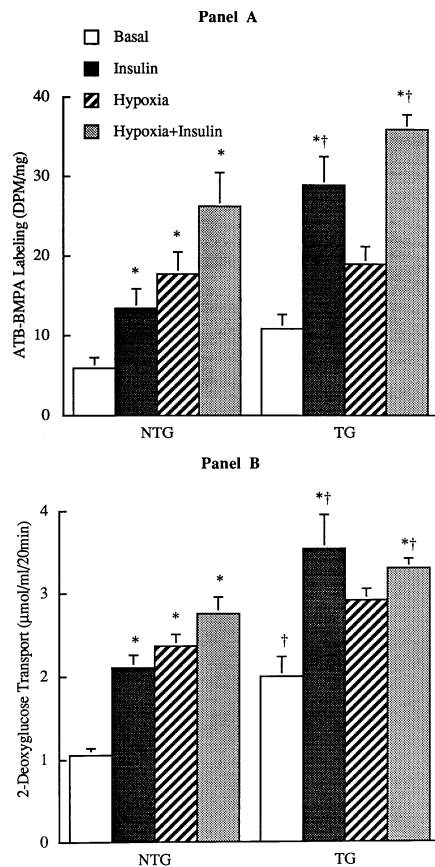
Figure 2   ATB-BMPA GLUT4 labelling (A) and 2DG transport (B) in isolated EDL muscles under basal conditions and on stimulation with insulin, hypoxia and hypoxia plus insulin

EDL muscles from fed NTG and TG mice were isolated and incubated as described in the Experimental section. For TG mice both hemizygous and homozygous mice were used; preliminary observations indicated that there was no difference in the responses of the muscles of the two groups and therefore the data were pooled. For photolabelling, $n = 6$ for NTG and $n = 7$ for TG mice. For 2DG transport, $n = 12$ for NTG and $n = 15$ for TG mice. Values are means $\pm$ S.E.M. *Significantly different from basal ($P < 0.05$); †significantly different from corresponding NTG value ($P < 0.05$).

The characteristics of membranes isolated from NTG and TG mice are given in Table 3. The membrane isolation procedure utilized ~ 1.0 g of muscle, and yielded ~ 170 μg of plasma membrane protein and ~ 140 μg of intracellular membrane protein. The enrichment of the plasma membrane marker enzyme KpNPPase showed that the plasma membrane fractions for both the NTG and TG mice were enriched ~ 22-fold in plasma membrane protein, while intracellular membranes were enriched ~ 8-fold over the crude homogenate in KpNPPase activity. KpNPPase yields averaged $\sim 2.7\%$ in the plasma membrane fraction and 1.1% in the intracellular membrane fraction. The enrichment and yield of this plasma membrane marker enzyme did not differ between NTG and TG mice, and was not affected by insulin/contraction treatment.

Membrane GLUT4 values are also shown in Table 3. Under basal conditions, skeletal muscle plasma membrane GLUT4 was approx. 4-fold greater in TG than in NTG mice. Insulin/contraction treatment significantly increased plasma membrane GLUT4 in both NTG and TG mice (2.4- and 1.4-fold increases respectively). Basal intracellular membrane GLUT4 was approx. 2-fold higher in TG than in NTG mice, and insulin/contraction

Table 3   Characteristics of skeletal muscle plasma membrane and intracellular membranes from NTG and TG $db/+$ mice in the basal state and in response to insulin plus contraction

Values are means $\pm$ S.E.M. of the numbers of measurements in parentheses *, Significantly different from basal; †, significantly different from corresponding NTG value ($P < 0.05$). PM, plasma membrane; IM, intracellular membranes. Fold enrichments are enzyme specific activities in the membrane fraction relative to those in the crude homogenate. The GLUT4 content is expressed as a percentage of that in a 50 μg sample of rat skeletal muscle intracellular membrane protein.

Parameter	NTG mice		TG mice	
	Basal ($n = 7$)	Insulin/contraction ($n = 4$)	Basal ($n = 7$)	Insulin/contraction ($n = 6$)
Tissue weight (mg)	934 ± 26	976 ± 30	1014 ± 20	1085 ± 47
Protein yield				
Homogenate (mg)	109 ± 9	155 ± 8	118 ± 6	138 ± 8
PM (μg)	162 ± 33	189 ± 15	160 ± 31	187 ± 19
IM (μg)	153 ± 35	115 ± 11	143 ± 21	130 ± 15
KpNPPase enrichment (fold)				
PM	23.1 ± 9.6	16.3 ± 2.6	25.7 ± 8.1	20.0 ± 4.5
IM	7.9 ± 2.9	4.6 ± 1.5	10.9 ± 2.6	8.5 ± 2.3
KpNPPase yield (%)				
PM	2.8 ± 0.7	1.9 ± 0.1	2.8 ± 0.5	2.8 ± 0.9
IM	1.1 ± 0.3	0.31 ± 0.08	1.2 ± 0.3	0.67 ± 0.16
GLUT4 (% of IM standard)				
PM	30.6 ± 3.6	$74.9 \pm 6.6^*$	$129.4 \pm 13.3†$	$182.8 \pm 16.8^*†$
IM	89.6 ± 4.9	63.4 ± 8.4	$210.0 \pm 11.0†$	$163.1 \pm 8.2^*†$

reduced intracellular membrane GLUT4 by 30% and 20% in NTG and TG mice respectively. The intracellular membrane GLUT4 levels were significantly higher in TG than in NTG mice under all conditions.

Photolabelling of isolated EDL muscles with ATB-BMPA is shown in Figure 2(A). Insulin, hypoxia and hypoxia plus insulin all increased cell-surface-labelled GLUT4 in EDL muscles from both NTG and TG mice. The increase with hypoxia in TG mice, however, was not statistically significant. Under basal conditions, and in response to insulin and hypoxia plus insulin, cell-surface-labelled GLUT4 was greater in EDL muscles from TG than from NTG mice. However, these differences were statistically different only in the insulin- and insulin plus hypoxia-stimulated states. In contrast, cell-surface-labelled GLUT4 in EDL muscles was not significantly different between NTG and TG mice during stimulation with hypoxia alone.

DISCUSSION

The purpose of the present study was to characterize further the effects of GLUT4 overexpression on GLUT4 subcellular distribution and its relationship with insulin- and contraction-stimulated glucose transport in skeletal muscle. Concurrent with this aim, it was necessary to characterize more fully the hGLUT4 TG mice produced in the inbred C57bl/KsJ-$m + /db$ background so that legitimate comparison with other strains of NTG and GLUT4 TG mice could be made. Our results indicate that the hGLUT4$db/+$ line 8 mice are similar to the hGLUT4B and hGLUT4C mice that were characterized previously, although the phenotype may be somewhat less pronounced due to a lower GLUT4 overexpression level (~ 3-fold compared with ~ 10-fold).

In hGLUT4$db/+$ line 8 mice, both fed and fasted plasma glucose levels were significantly decreased, as has been reported

with other lines of GLUT4 TG mice [9,10,12]. Furthermore, a marked enhancement of the ability to dispose of a glucose load following an oral challenge was observed in the TG mice compared with NTG controls (Figure 1). These results are comparable with our previous findings with hGLUT4B and hGLUT4C TG mice, although the NTG $db/+$ mice have a slightly lower fasting plasma glucose and a much diminished glucose excursion following the challenge compared with NTG mice on hybrid backgrounds; the NTG $db/+$ mice typically reach a peak plasma glucose of only $\sim$ 140–150 mg/dl, whereas NTG hybrid mice reach a peak of $\sim$ 200–300 mg/dl in similar tests [12]. As observed in hGLUT4B and hGLUT4C TG mice, fed plasma insulin levels were lower and fed plasma glucagon levels were higher in TG mice compared with NTG mice [10]; however, unlike in the earlier study, we failed to detect a significant rise in plasma glucagon in the TG mice following an overnight fast.

Glycogen levels in skeletal and cardiac muscles, but not liver, were elevated in hGLUT4$db/+$ TG mice compared with NTG $db/+$ mice, which is similar to what was observed previously in hGLUT4B TG mice. However, the magnitude of this increase was less in the present study than in the earlier study; here, increases of 1.2-fold (not significant) and 2-fold ($P < 0.05$) were observed for the diaphragm and quadriceps respectively, compared with values of 4-fold and 3-fold in hGLUT4B mice [10]. Similarly, cardiac glycogen was increased 4-fold in hGLUT4$db/+$ TG mice and 5-fold in hGLUT4B TG mice [10] compared with the respective NTG controls. The diminished magnitude of the effects on glycogen accumulation in the present study are entirely consistent with the lower level of GLUT4 overexpression in the mice used here compared with previous studies ($\sim$ 3-fold and $\sim$ 10-fold respectively).

Interestingly, total plasma cholesterol was significantly decreased in hGLUT4$db/+$ TG mice compared with NTG $db/+$ mice (Table 2). Analysis of the cholesterol profile indicated that both the low-density and high-density lipoprotein fractions were decreased in the TG mice, whereas the very-low-density lipoprotein fraction was not altered. In an earlier study [10], total plasma cholesterol in female hGLUT4 (lines B and C) mice was not different from levels in NTG mice. Similarly, in the present study, total plasma cholesterol was not different in female hGLUT4$db/+$ TG mice compared with NTG $db/+$ mice (results not shown), which suggests that a hormonal factor may influence the effect of the GLUT4 transgene on plasma cholesterol.

Taken together, the metabolic and physiological profiles of the hGLUT4$db/+$ TG mice are qualitatively similar to those of the previously characterized lines of GLUT4 TG mice, although there are quantitative differences, some of which can be accounted for by the lower level of GLUT4 overexpression and others by the different genetic background. Our assessment is that these mice are suitable for characterization of GLUT4 trafficking under various stimuli, and should give results comparable with those that would be obtained with the hybrid GLUT4 TG mice. Furthermore, this line of mice has the important advantage that, in future studies, the effects of diabetes can be assessed by using littermates that are homozygous for the db gene.

Previously we have attempted to investigate the relationship between GLUT4 overexpression and cell surface GLUT4 in TG mice in the basal or insulin-stimulated state by immunofluorescence or membrane fractionation [10,13]. Both of these techniques indicated that large numbers of GLUT4 transporters were associated with the plasma membrane in the basal state. However, neither of these techniques was able to distinguish between active and inactive GLUT4 transporters. Therefore, in the present study we assessed the GLUT4 subcellular distribution using the membrane-impermeant glucose transporter photolabelling compound ATB-BMPA, which only labels surface-accessible, active, glucose transporters [15–17].

Western blotting of isolated plasma membranes from NTG and TG mice showed a markedly increased GLUT4 content ($\sim$ 3-fold) in the latter in both the basal and insulin/contraction-stimulated states. These results are similar to previous membrane isolation [13] and immunofluorescence [4,9] data from GLUT4 TG mice which indicated a marked increase in plasma membrane GLUT4 in the basal state. Together, these findings suggest that the overexpression of GLUT4 has saturated the intracellular GLUT4 compartment or overloaded the vesicular trafficking pathway, resulting in an increase in GLUT4 associated with the plasma membrane in the basal state.

With regard to membrane fractionation, it should be pointed out that the intracellular membranes contain a large proportion of T-tubule membranes which are of cell surface origin and have been shown to be a site for GLUT4 translocation in response to insulin [13,24]. More recent data from our laboratory (J. T. Brozinick, T. H. Reynolds and S. W. Cushman, unpublished work) has shown that insulin stimulation increases GLUT4 photolabelling in T-tubule membranes, indicating that the T-tubules are active sites for glucose transport. Therefore, when only the plasma membrane fraction is considered, membrane fractionation actually underestimates the total amount of GLUT4 that is associated with the cell surface. However, when all of the cell surface GLUT4 is considered (plasma membrane and T-tubules which are in the intracellular membranes), the discrepancy between cell surface GLUT4 on the one hand, and basal and insulin-stimulated glucose transport on the other, is actually much greater than originally believed.

In qualitative agreement with the membrane isolation data, photolabelling of isolated EDL muscles showed an increase in basal cell surface GLUT4 in the TG mice. As suggested previously, this indicates that overexpression of GLUT4 in skeletal muscle may 'flood' the vesicular trafficking pathways and overcome the normal intracellular sequestration of GLUT4 under basal conditions, despite the hypoinsulinaemia which would presumably reduce the signal that initiates translocation. Similarly, previous studies have shown that increasing GLUT4 expression through exercise training results in an increase in the amount of GLUT4 associated with the plasma membrane fraction in the basal state [7,25,26].

Qualitatively, however, the fold increase in basal plasma membrane GLUT4 in the TG mice as assessed by Western blotting of membrane fractions ($\sim$ 4-fold) is much larger than that assessed by photolabelling ($\sim$ 2-fold). Nevertheless, the increase in basal 2DG transport in the TG mice parallels the increase in cell surface GLUT4 photolabelling. One possible explanation for this discrepancy is that disruption of the normal vesicular trafficking pathways by overexpression of GLUT4 may have caused glucose transporters to become associated with the plasma membrane in a state in which they are unable to bind substrate, but they are still isolated with the plasma membrane during the fractionation procedure. Alternatively, the much larger increase in plasma membrane GLUT4 with Western blotting could be due to cross-contamination by intracellular membrane GLUT4. Which of these possibilities is correct remains to be determined by future studies.

Stimulation of isolated EDL muscles by insulin, hypoxia or hypoxia plus insulin resulted in significant increases in cell surface GLUT4 photolabelling in both NTG and TG mice. These results again are qualitatively similar to those obtained here and previously [13] using membrane isolation and Western blotting. However, unlike the results from membrane isolation

experiments, which show greater increases in plasma membrane GLUT4 than in glucose transport activity in the isolated muscles, the fold increases in cell surface photolabelling match the increases in 2DG transport under each of these conditions. Thus, as noted above, under basal conditions it appears that the membrane fractionation procedure is compromised either by isolation of GLUT4 molecules which are associated with the plasma membrane but do not participate in glucose transport, or by cross-contamination of plasma membrane GLUT4 by intracellular membrane GLUT4. In addition, there may be a limitation to the amount of GLUT4 that can be inserted into the plasma membrane [13]. Thus, although the overexpressed GLUT4 transporters may be able to be translocated to the plasma membrane, all of the GLUT4-containing vesicles may not be able to dock and fuse with it, and hence would be unable to transport glucose.

It is interesting to note that the overexpression of GLUT4 does not have an appreciable effect on the hypoxia/contraction stimulus pathway. This contrasts with the data of Hansen et al. [9], which showed an increase in contraction-stimulated glucose transport activity in EDL muscles from another line of GLUT4 TG mice. The reason for this difference is not readily apparent, but the EDL muscles in the study of Hansen et al. [9] were stimulated *in situ*, whereas we used hypoxia *in vitro* to mimic the action of muscle contraction. Since Hansen et al. [9] used *in situ* contraction, it is possible that the degree of stimulation of 2DG transport they observed with contraction was actually in part an additive effect of contraction plus the insulin present in the circulation [4]. A considerable amount of previous data imply the existence of insulin- and contraction-sensitive pools of GLUT4 in skeletal muscle [2,3,27]. In light of the lack of an effect of GLUT4 overexpression on contraction-stimulated glucose transport activity, it is of interest to note that exercise-trained white gastrocnemius muscles show an increase in total GLUT4, yet no increase in contraction-stimulated glucose transport activity [7,26]. Thus it is possible that, in the mice used in the present study, the overexpressed GLUT4 is contained only in an insulin-sensitive pool.

In the NTG mice, the increases in 2DG transport and cell surface GLUT4 photolabelling during stimulation by the combination of hypoxia plus insulin were significantly greater than with either stimulus alone. These results provide strong support for the recent observations ([17,28]; C.M Wilson, J. T. Brozinick and S. W. Cushman, unpublished work) that the additive effects of hypoxia (or muscle contraction) and insulin on glucose transport are due to an additive increase in cell surface GLUT4. These results contrast with previous membrane isolation studies which did not show such additivity [1,2]. The present results support the concept that the subcellular fractionation approach is hampered by the inability to distinguish between active and inactive GLUT4 glucose transporters, as well as by the significant problems of cross-contamination of plasma membrane GLUT4 by intracellular membrane GLUT4 and incomplete isolation of all cell surface membranes in the plasma membrane fraction.

In conclusion, the present study demonstrates that overexpression of GLUT4 in mouse skeletal muscle results in an increase in glucose transport on stimulation by insulin or hypoxia plus insulin, but not by hypoxia alone. Despite the 3-fold overexpression of GLUT4 in the TG mice, the increases in glucose transport activity are modest (1.7- and 1.2-fold with insulin and hypoxia plus insulin respectively). The increases in transport were mirrored quantitatively by increases in cell surface

GLUT4 and qualitatively by GLUT4 detected in isolated plasma membranes by Western blotting. However, the greater increases in plasma membrane GLUT4 than in cell surface GLUT4 in the TG mice indicate either that the plasma membrane fraction is contaminated with intracellular membrane GLUT4 or that some of the GLUT4 isolated with the plasma membranes is nonfunctional. These observations indicate that overexpression of GLUT4 in skeletal muscle may occur without simultaneous increases in the proteins necessary for the trafficking of GLUT4 vesicles and/or for intracellular glucose disposal, thus resulting in only modest increases in glucose transport. Furthermore, our results indicate that, at least under the experimental conditions utilized here, GLUT4 overexpression chiefly affects the insulin-stimulated glucose transport pathway.

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