Glucose transport and GLUT4 protein distribution in skeletal muscle of GLUT4 transgenic mice

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The aim of the present investigation was to determine whether the subcellular distribution and insulin-stimulated translocation of the GLUT4 isoform of the glucose transporter are affected when GLUT4 is overexpressed in mouse skeletal muscle, and if the overexpression of GLUT4 alters maximal insulin-stimulated glucose transport and metabolism. Rates of glucose transport and metabolism were assessed by hind-limb perfusion in GLUT4 transgenic (TG) mice and non-transgenic (NTG) controls. Glucose-transport activity was determined under basal (no insulin), submaximal (0.2 m-unit/ml) and maximal (10 m-units/ml) insulin conditions using a perfusate containing 8 mM 3-*O*-methyl-D-glucose. Glucose metabolism was quantified by perfusing the hind limbs for 25 min with a perfusate containing 8 mM glucose and 10 m-units/ml insulin. Under basal conditions, there was no difference in muscle glucose transport between TG (1.10 ± 0.10 μmol/h per g; mean $\pm$ S.E.M.) and NTG (0.93 ± 0.16 μmol/h per g) mice. However, TG mice

displayed significantly greater glucose-transport activity during submaximal (4.42 ± 0.49 compared with 2.69 ± 0.33 μmol/h per g) and maximal (11.68 ± 1.13 compared with 7.53 ± 0.80 μmol/h per g) insulin stimulation. Nevertheless, overexpression of the GLUT4 protein did not alter maximal rates of glucose metabolism. Membrane purification revealed that, under basal conditions, plasma-membrane (~ 12-fold) and intracellular-membrane (~ 4-fold) GLUT4 protein concentrations were greater in TG than NTG mice. Submaximal insulin stimulation did not increase plasma-membrane GLUT4 protein concentration whereas maximal insulin stimulation increased this protein in both NTG (4.1-fold) and TG (2.6-fold) mice. These results suggest that the increase in insulin-stimulated glucose transport following overexpression of the GLUT4 protein is limited by factors other than the plasma-membrane GLUT4 protein concentration. Furthermore, GLUT4 overexpression is not coupled to glucose-metabolic capacity.

INTRODUCTION

Skeletal muscle is believed to be the main tissue responsible for insulin-stimulated glucose clearance *in vivo*, and it is a major site of insulin resistance in non-insulin-dependent diabetes mellitus. Mediating skeletal-muscle glucose uptake is the insulin-regulatable glucose transporter, GLUT4. Under basal conditions, the GLUT4 transporter is sequestered in a unique intracellular vesicular compartment [1,2]. In response to insulin, these GLUT4-containing vesicles are translocated to and fuse with the plasma membrane resulting in an increased rate of glucose transport [3–6]. It has been demonstrated in skeletal muscle that there is a strong correlation between total GLUT4 protein concentration and glucose transport [7–9]. Despite the fact that insulin resistance in skeletal muscle does not appear to be due to a decrement in total GLUT4 protein concentration, evidence exists that treatments that increase muscle GLUT4 protein concentration ameliorate some of the effects of insulin resistance on muscle glucose transport [10–12]. Hence modalities that increase skeletal-muscle GLUT4 protein concentration could be of therapeutic value in treating non-insulin-dependent diabetes mellitus.

Recently, a strain of transgenic (TG) mice that displays an approximately 12-fold overexpression of the human GLUT4 gene in a tissue-specific (adipose, heart and skeletal muscle) manner has been developed [13,14]. These animals are hypo-glycaemic and hypoinsulinaemic relative to non-transgenic (NTG) mice, and demonstrate an increased ability to dispose of

an oral [13] or intravenous [15] glucose load. Furthermore, the soleus muscles of these animals exhibit enhanced basal and insulin-stimulated glucose uptake *in vitro*. Immunofluorescence data on soleus muscles suggest that these increases in basal and insulin-stimulated glucose uptake are accompanied by an increase in GLUT4 protein associated with the plasma membrane [15]. However, immunofluorescence does not provide enough resolution to distinguish between plasma- and subplasma-membrane glucose transporters, especially in a tissue where the majority of the cell volume is occupied by the microfibrils thus relegating most of the cytoplasm to a region close to the cell surface. Therefore the purpose of the present study was to examine the subcellular distribution and insulin-stimulated translocation of GLUT4 protein in the skeletal muscle of these TG mice by membrane fractionation, and to determine the relationship between GLUT4 protein overexpression and insulin-stimulated glucose transport and metabolism.

EXPERIMENTAL

Animals

Some 23 TG mice carrying 11.5 kb (*Kpn*I–*Eco*RI fragment) of the human GLUT4 transporter genomic DNA (hGLUT-4-11.5B) as described by Olson et al. [14] and 23 age-matched NTG littermates were used in this investigation. The animals were randomly assigned to a glucose-transport (36) or glucose-disposal

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(10) group. The glucose-transport group was further subdivided into those receiving no insulin (basal), those receiving sub-maximal insulin and those receiving maximal insulin. The NTG and TG mice were balanced in each group. The mice were housed in individual cages in a temperature-controlled animal room (21 °C) maintained on a 12 h light/12 h dark cycle. They were provided with normal mouse chow and water *ad libitum* throughout the study.

Surgical preparation and hind-limb perfusion

After 6 h of food deprivation, the mice were anaesthetized with an intraperitoneal injection (0.3 ml) of a 1:10 dilution of Innovar-Vet (Pitman-Moore, Mundelein, IL, U.S.A.) followed by 0.1 ml of diazepam (5 mg/ml; Elkins-Sinn, Cherry Hills, NJ, U.S.A.) per 30 g of body weight. The surgical procedure and perfusion apparatus were similar to those previously described by Ruderman et al. [16]. After the mouse had been prepared for cannulation, 100 units of heparin was injected via the abdominal vena cava to prevent clotting of blood in the capillary beds of the hind-limb muscles. Catheters were then inserted into the abdominal aorta and vena cava, and the hind limbs were washed out with 8 ml of Krebs–Henseleit buffer, pH 7.4. During this wash-out period the mouse was killed with an intracardiac injection of sodium pentobarbital. The catheters were then placed in line with a non-recirculating perfusion system, which provided a constant flow rate of 1.8 ml/min, and continuous gassing (95 % O_2/5 % CO_2) of the perfusate.

For measurement of glucose transport, the hind limbs were allowed to stabilize during a 10 min wash-out period. The perfusate, warmed to 37 °C, consisted of Krebs–Henseleit buffer, pH 7.4, containing 4 % dialysed BSA (Cohn fraction V; U.S. Biochemical Corp., Cleveland, OH, U.S.A.), 15 % washed time-expired human erythrocytes, 0.2 mM pyruvate and 1.0 mM glucose. After the initial 10 min wash-out period, basal glucose transport was measured over an 8 min period using an 8 mM concentration of the non-metabolizable glucose analogue 3-*O*-methyl-D-glucose (3-MG) (48 μCi of 3-[^{3}H]MG/mmol) and 2 mM mannitol (80 μCi of D-[1-^{14}C]mannitol/mmol). For maximal and submaximal insulin-stimulated glucose transport, 10 m-units/ml and 0.2 m-unit/ml insulin respectively were added to the perfusate. Perfusion time for the insulin-stimulated groups was 6 min. At the end of the perfusion, the triceps surae group was removed from each leg, blotted on gauze dampened in cold Krebs–Henseleit buffer and clamp-frozen in tongs cooled in liquid N_2. The remaining muscle from the hind limb was then immediately removed, cleaned of fat and connective tissue and frozen in liquid N_2. The triceps surae group from the right leg was used to determine 3-MG transport and the triceps surae group from the left leg was used to determine hexokinase activity. The triceps surae group was selected because its muscle fibre composition is representative of the hind-limb fibre composition [17]. The pooled muscle from the hind limbs was used for purification of plasma and microsomal membranes (see below). The muscles were stored at −80 °C until analysed.

For measurement of glucose metabolism and disposal, mice were prepared for hind-limb perfusion as described above, cannulated and placed in line with the non-recirculating perfusion system. The hind limbs were allowed to equilibrate for 10 min with perfusate containing Krebs–Henseleit buffer, pH 7.4, 4 % dialysed BSA, 15 % washed time-expired human erythrocytes, 0.2 mM pyruvate, 0.5 mM glucose and 10 m-units/ml insulin. After the equilibration period the perfusate was changed to one containing 8 mM glucose (0.25 μCi/ml D-[U-^{14}C]glucose). The [^{14}C]glucose was added to determine rates of glucose incor-

poration into glycogen and glucose oxidation. At the completion of the 25 min perfusion, the triceps surae group was removed from each leg, blotted on gauze dampened with cold Krebs–Henseleit buffer, clamp-frozen in liquid N_2 and stored at −80 °C until analysis of glucose incorporation into glycogen.

Determination of average 3-MG transport

Muscle samples were weighed, homogenized in 1 ml of 10 % trichloroacetic acid at 4 °C and centrifuged in a microcentrifuge (Fisher Scientific, Houston, TX, U.S.A.) for 10 min. Duplicate 0.3 ml samples of the supernatant were transferred to 7 ml scintillation vials containing 6 ml of Bio-Safe II (Research Products International Corp., Mount Prospect, IL, U.S.A.) and vortexed. For determination of perfusate specific radioactivity, well-mixed samples of the arterial perfusate were obtained during the perfusion. The samples were deproteinized in 10 % trichloroacetic acid and treated the same as the muscle homogenates. The samples were counted for radioactivity in an LS-6000 liquid-scintillation spectrophotometer (Beckman, Fullerton, CA, U.S.A.). The accumulation of intracellular 3-[^{3}H]MG, which is indicative of muscle glucose transport, was calculated by subtracting its concentration in the extracellular space from its total muscle concentration. The 3-[^{3}H]MG in the extracellular space was quantified by measuring the concentration of [^{14}C]mannitol in the muscle homogenate.

Determination of glucose metabolism and disposal

Total muscle glucose metabolism was calculated from the arteriovenous difference, the perfusate flow rate and the weight of the muscle perfused. Perfusate glucose concentration was determined with a 23A glucose analyser (YSI, Yellow Springs, IL, U.S.A.). The rate of glucose oxidation was determined from the arteriovenous difference in $^{14}CO_2$ and flow rate. To determine the $^{14}CO_2$ content of the arterial and venous perfusate, duplicate 2.5 ml samples of arterial perfusate were obtained from the perfusate reservoir and 2.5 ml samples of venous effluent samples were obtained by syringe through a rubber adapter on the venous line at 22 min of perfusion. The perfusate samples were immediately injected into 25 ml flasks fitted with serum caps and hanging centre wells. The perfusate samples were acidified with 0.5 ml of 1.0 M acetic acid and the released $^{14}CO_2$ trapped on a strip of filter paper located in the centre well soaking in 0.4 ml of Protosol (New England Nuclear, Boston, MA, U.S.A.). The acidified perfusate was allowed to stand overnight. The filter paper strips and Protosol were then quantitatively transferred to a scintillation vial containing 6 ml of Bio-Safe II, and radioactivity was determined by scintillation spectrophotometry.

Lactate concentration was determined from samples obtained from the arterial reservoir and venous effluent which was collected on ice during the 25 min perfusion. The samples were deproteinized in 8 % perchloric acid and centrifuged. Lactate was determined on the acid extracts by the method of Hohorst [18]. Lactate accumulation was calculated from the arteriovenous difference, perfusate flow rate and the weight of the muscle perfused.

The rate of [^{14}C]glucose incorporation into glycogen was determined by isolating total muscle glycogen as described by Lo et al. [19]. The triceps surae group was weighed frozen and then placed into test tubes containing 1 ml of 30 % KOH saturated with Na_2SO_4 and digested by incubating the tubes for 30 min at 100 °C. After the 30 min incubation, the tubes were cooled to room temperature, 1.2 vol. of 95 % ethanol was added and the glycogen was allowed to precipitate overnight at −20 °C. The

glycogen was pelleted by centrifugation (1200; g 30 min; 4 °C) and resuspended in distilled water. An aliquot of the suspension was transferred to a scintillation vial containing 6 ml of Bio-Safe II, and radioactivity was determined.

Plasma- and intracellular-membrane preparations

Plasma and intracellular membranes (low-density microsomes) were prepared from approx. 1.5 g of frozen hind-limb muscles by a modification of the procedure of Hirshman et al. [6]. The muscle was pulverized in a mortar and pestle under liquid N_2. The pulverized muscle was homogenized using a Polytron PT-10 homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.) at slow speed in 10 ml of buffer consisting of 255 mM sucrose, 100 mM Tris/HCl, pH 7.6 and 0.2 mM EDTA, then further homogenized with a Potter–Elvehjem Teflon/glass 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 content, and the remainder of the homogenate was centrifuged at 34 000 g for 20 min. The resulting pellet was used for purification of plasma membranes and the supernatant was used for purification of intracellular membranes.

Plasma membranes were purified by resuspending the pellet in a buffer consisting of 250 mM sucrose and 20 mM Hepes, pH 7.4. To dissolve myofibrillar protein, concentrated KCl and sodium pyrophosphate were added to the suspension resulting in a final concentration of 300 mM and 25 mM respectively. This solution was mixed and centrifuged at 227 000 g for 60 min in a Beckman 70.1Ti rotor. The supernatant was discarded and the pellet was resuspended in sucrose/Hepes buffer. DNase (Worthington Enzymes, Freehold, NJ, U.S.A.) and protease inhibitors (aprotinin, leupeptin, pepstatin) were then added at concentrations of 2000 Kunitz units per ml and 2.5 μg/ml respectively. The mixture was incubated at 30 °C for 60 min, diluted 1:2 with ice-cold sucrose/Hepes buffer and centrifuged at 227 000 g for 10 min in a Beckman TLA100.4 rotor. The resulting pellet was resuspended in 34 % buffered sucrose and layered on top of 45 and 38 % sucrose layers. Sucrose layers of 32, 30, 27 and 8 % were layered on top to complete the gradient. The gradient was centrifuged in a Beckman SW-29.1Ti swinging-bucket rotor at 68 000 g for 16 h. The 12/27 and 27/30 % interfaces, which contained the plasma membranes, were removed, diluted with 20 mM Hepes, and centrifuged at 331 000 g for 60 min in a 70.1Ti rotor. The resulting pellet was resuspended in sucrose/Hepes buffer to give a final protein concentration of 1–2 mg/ml.

Intracellular membranes were prepared from the supernatant of the initial 34 000 g spin. The supernatants were centrifuged at 227 000 g for 60 min in a Beckman 70Ti rotor. The pellet was resuspended in a buffer containing 155 mM Tris/HCl, pH 8.2, and 0.2 mM EDTA, and centrifuged at 227 000 g for 7 min in a Beckman TLA100.4 rotor. The resulting pellet was resuspended in a buffer consisting of 255 mM sucrose, 1.0 mM Tris/HCl, pH 8.5, and 1 mM $MgCl_2$. This suspension was centrifuged at 227 000 g for 7 min in a TLA 100.4 rotor and the resulting pellet resuspended in 1 ml of water and layered over a discontinuous 0.9 M sucrose gradient in 20 mM Tris/HCl, pH 7.4/1 mM EDTA. The gradient was centrifuged at 135 000 g for 22 min in a Beckman TLS-55 swinging-bucket rotor. The sucrose/buffer interface and buffer above the interface were removed, diluted in water and centrifuged at 331 000 g for 14 min in a TLA-100.4 rotor. The resulting pellet was resuspended in sucrose/Hepes buffer to give a final protein concentration of 1–2 mg/ml.

Each intracellular- and plasma-membrane suspension was weighed to determine the exact volume. An aliquot of each suspension was removed for marker enzyme and protein deter-minations. The remaining suspension was used for Western blotting. All samples were stored at -70 °C until analysed.

Assays of protein and marker enzymes

Homogenate and membrane protein were determined for each preparation by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.) with crystalline BSA as standard. K^+-stimulated p-nitrophenol phosphatase (KpNPPase) specific activity was measured as a marker for plasma membranes by the method of Bers et al. [20].

Western blotting of plasma- and intracellular-membrane GLUT-4 protein

Plasma- and intracellular-membrane (30 μg of protein) samples along with molecular-mass markers (Gibco/BRL, Bethesda, MD, U.S.A.) were subjected to SDS/PAGE under reducing conditions using a 12 % resolving gel. 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.), using a Bio-Rad SD semidry transfer unit. After transfer, the PVDF sheets were blocked in Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7.5/500 mM NaCl) containing 5 % non-fat dried milk, pH 7.5, at 37 °C. Next, the sheets were washed in TBS with 0.05 % Tween 20 (TTBS) for 20 min and incubated overnight with the monoclonal GLUT4 antibody 1F8 (donated by Dr. Paul Pilch, Boston University School of Medicine, Boston, MA, U.S.A.), the monoclonal dihydropyridine (DHP) receptor antibody IIID5 E1 (donated by Dr. Kevin Campell, Howard Hughes Medical Institute, University of Iowa, Aimes, IA, U.S.A.) or the monoclonal antibody MCB2 raised against the α_1-subunit of $(Na^+ + K^+)$-ATPase (donated by Dr. Kathleen Sweadner, Harvard University, Boston, MA, U.S.A.) in TTBS, containing 1 % milk and 0.02 % NaN_3. The PVDF sheets were then washed in TTBS for 20 min followed by a 1 h incubation with 0.25 μCi/ml ^{125}I-labelled sheep anti-mouse IgG (New England Nuclear, Boston, MA, U.S.A.), 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 areas of each band were expressed as a percentage of a standard (100 μg of rat skeletal-muscle microsomal membrane protein) run on each gel.

Hexokinase activity

Muscle samples were homogenized (1:40, w/v) in 25 mM KF/20 mM EDTA buffer, pH 7.0. Hexokinase activity was determined by the procedure of Lowry [21].

Statistical analysis

The data were analysed using a one-way analysis of variance. When a significant F ratio was obtained, a Newman–Keuls *post hoc* test was utilized to identify differences between the means, with significance being set at $P < 0.05$.

RESULTS

Skeletal-muscle glucose transport

The rate of skeletal-muscle glucose transport was determined under conditions of basal (no insulin), submaximal and maximal insulin stimulation (Table 1). No difference was observed under basal conditions between NTG and TG mice. During submaximal and maximal insulin stimulation both groups of mice exhibited

Table 1   Rates of 3-MG transport for NTG and TG mice under basal, submaximal (0.2 m-unit/ml) and maximal (10.0 m-units/ml) insulin stimulation

Values are means ± S.E.M. Numbers in parentheses are the number of mice per group.

	Rate (μmol/h per g)		
	Basal	Submaximal insulin	Maximal insulin
NTG mice	0.93 ± 0.16 (5)	2.69 ± 0.33* (7)	7.53 ± 0.80* (8)
TG mice	1.10 ± 0.09 (5)	4.42 ± 0.49* (7)	11.68 ± 1.13*† (8)

* Significantly different from basal ($P < 0.05$).
† Significantly different from NTG ($P < 0.05$).

increased rates of glucose transport when compared with basal conditions. The TG mice displayed significantly greater rates of glucose transport under both submaximal (64 %) and maximal (55 %) insulin stimulation than the NTG mice.

Glucose metabolism

In the presence of 8 mM glucose and 10 m-units/ml insulin, the rates of glucose metabolism were found to be similar in NTG and TG mice (Table 2). There were no differences in [^{14}C]glucose

incorporation into glycogen, lactate accumulation or glucose oxidation between TG and NTG mice during maximal insulin stimulation. However, muscle hexokinase activity was significantly greater in NTG than in TG mice.

Membrane isolation and characterization

The membrane-isolation procedure used an average of 1.52 g of muscle and yielded ∼ 282 μg of plasma-membrane protein and ∼ 257 μg of intracellular-membrane protein (Table 3). The purity and yield of the plasma-membrane marker KpNPPase are presented in Table 4. The relative enrichment of the plasma-membrane markers KpNPPase and ($Na^+ + K^+$)-ATPase and the T-tubule-membrane marker, DHP receptor, are given in Table 5. On the basis of the KpNPPase activity, plasma membranes were enriched an average of 30-fold over the crude homogenate. KpNPPase yields averaged 4.6 % in the plasma-membrane fraction and 1.3 % in the intracellular-membrane fraction. Furthermore, both plasma-membrane markers, KpNPPase and ($Na^+ + K^+$)-ATPase, were enriched approximately 3-fold in the plasma-membrane fraction relative to the intracellular membranes, and the DHP receptor was enriched 5.3-fold in the intracellular membranes relative to the plasma membranes. Protein yield, fold enrichment and percentage yield did not differ between NTG and TG mice and was not affected by insulin treatment. Furthermore, the distributions of the membrane markers in membranes isolated from mouse muscle were identical with those of membranes previously isolated from rat skeletal muscle.

Table 2   Rates of total glucose metabolism, [^{14}C]glucose incorporation into glycogen, lactate accumulation, glucose oxidation and hexokinase activity in NTG and TG mice during perfusion with 8 mM glucose and 10 m-units/ml insulin

Values are means ± S.E.M. Five TG and five NTG mice were used to determine glucose metabolism, glycogen synthesis, lactate accumulation and glucose oxidation. Eight TG and eight NTG mice were used for hexokinase determination.

	Glucose metabolism (μmol/h per g)	Glycogen synthesis (μmol/h per g)	Lactate accumulation (glucose equiv.) (μmol/h per g)	Glucose oxidation (nmol/h per g)	Hexokinase activity (μmol/min per g)
NTG	23.94 ± 3.49	8.96 ± 1.72	8.69 ± 1.43	208.0 ± 23.00	6.42 ± 0.20
TG	25.56 ± 2.28	9.15 ± 0.86	11.10 ± 2.01	182.4 ± 24.47	5.32 ± 0.15*

* Significantly different from NTG ($P < 0.05$).

Table 3   Body and muscle weights used in membrane preparations and protein recovery

Values are means ± S.E.M. Numbers in parentheses are number of mice in each group. There were no differences between TG and NTG groups.

	Body weight (g)	Muscle weight (g)	Total protein (mg)		
			Homogenate	Plasma membrane	Intracellular membrane
NTG mice					
Basal (5)	30.1 ± 1.7	1.60 ± 0.05	220.3 ± 16.4	0.28 ± 0.03	0.28 ± 0.05
Submaximal insulin (7)	31.5 ± 1.0	1.32 ± 0.09	143.2 ± 8.3*	0.28 ± 0.02	0.20 ± 0.01
Maximal insulin (8)	29.2 ± 1.3	1.66 ± 0.08	212.6 ± 8.1	0.27 ± 0.03	0.28 ± 0.05
TG mice					
Basal (5)	30.7 ± 1.8	1.53 ± 0.14	211.1 ± 20.0	0.35 ± 0.05	0.30 ± 0.06
Submaximal insulin (7)	27.8 ± 1.0	1.35 ± 0.11	158.3 ± 11.9*	0.23 ± 0.04*	0.23 ± 0.02
Maximal insulin (8)	30.2 ± 1.2	1.66 ± 0.12	209.8 ± 8.9	0.28 ± 0.03	0.25 ± 0.04

* Significantly different from basal ($P < 0.05$).

Table 4 KpNPPase activities and percentage recoveries of membrane fractions under basal, submaximal and maximal insulin stimulation in skeletal muscle obtained from NTG and TG mice

Values are means $\pm$ S.E.M. Numbers in parentheses are number of mice in each group. There were no differences between TG and NTG groups.

	Homogenate specific activity (nmol/min per mg)	Plasma membrane		Intracellular membrane	
		Specific activity (nmol/min per mg)	Recovery (%)	Specific activity (nmol/min per mg)	Recovery (%)
NTG mice					
Basal (5)	4.71 ± 0.26	114.3 ± 10.7	3.31 ± 0.36	33.5 ± 5.1	0.94 ± 0.18
Submaximal insulin (7)	4.39 ± 0.36	129.3 ± 9.0	5.10 ± 0.64	48.9 ± 4.9*	1.44 ± 0.12
Maximal insulin (8)	3.73 ± 0.30	128.9 ± 10.3	4.51 ± 0.52	38.6 ± 3.3	1.48 ± 0.32
TG mice					
Basal (5)	3.98 ± 0.30	95.0 ± 12.8	4.15 ± 0.71	32.3 ± 5.6	1.07 ± 0.13
Submaximal insulin (7)	4.23 ± 0.38	157.7 ± 15.1*	5.94 ± 1.02	51.5 ± 5.2*	1.72 ± 0.25
Maximal insulin (8)	3.98 ± 0.28	135.2 ± 6.9*	4.57 ± 0.70	37.7 ± 3.4	1.08 ± 0.18

* Significantly different from basal ($P < 0.05$).

Table 5 Fold enrichment of plasma [KpNPPase and $(Na^+ + K^+)$-ATPase] and intracellular (DHP receptor) membrane markers under basal, submaximal and maximal insulin stimulation in skeletal muscle obtained from NTG and TG mice

Values are means $\pm$ S.E.M. Numbers in parentheses are number of mice in each group. There were no differences between TG and NTG groups.

	Fold enrichment					
	Plasma membrane			Intracellular membrane		
	KpNPPase	Na^+/K^+-ATPase	DHP receptor	KpNPPase	Na^+/K^+-ATPase	DHP receptor
NTG mice						
Basal (5)	24.2 ± 1.6	15.4 ± 1.8	21.9 ± 5.4	7.0 ± 0.7	7.0 ± 1.1	87.0 ± 15.2
Submaximal insulin (7)	30.8 ± 3.5	21.3 ± 3.1	8.0 ± 1.5	11.1 ± 0.6*	3.7 ± 0.3	93.8 ± 13.8
Maximal insulin (8)	35.3 ± 2.9	17.1 ± 3.3	18.8 ± 1.8	10.9 ± 1.4	7.8 ± 1.4	68.2 ± 12.9
TG mice						
Basal (5)	24.2 ± 3.2	15.0 ± 1.4	18.4 ± 6.4	8.2 ± 1.3	7.5 ± 1.2	81.2 ± 7.2
Submaximal insulin (7)	38.7 ± 4.6*	18.3 ± 4.7	7.1 ± 1.7	12.5 ± 1.2*	4.8 ± 0.9	119.8 ± 28.0
Maximal insulin (8)	34.4 ± 3.6*	17.9 ± 2.1	27.6 ± 5.4	9.7 ± 1.5	7.1 ± 0.9	92.0 ± 23.1

* Significantly different from basal ($P < 0.05$).
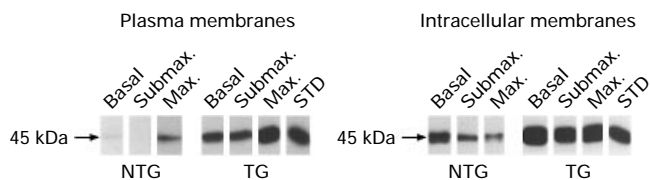
Figure 1 Autoradiogram showing GLUT4 protein bands in skeletal-muscle plasma and intracellular membranes from NTG and TG mice

Muscle was removed from the hind limbs after perfusion in the absence of insulin (basal) or after perfusion with a submaximal (0.2 m-unit/ml) or a maximal (10.0 m-units/ml) insulin concentration. The standard (STD) was 100 μg of rat skeletal-muscle microsomal membrane protein.

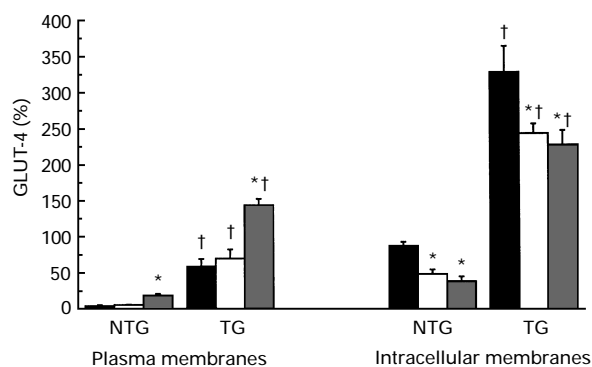## Plasma- and intracellular-membrane GLUT4 concentration

Plasma-membrane GLUT4 protein concentration was found to be approx. 12 times higher in TG mice than NTG mice under basal conditions (Figures 1 and 2). When stimulated with a submaximal insulin concentration, plasma-membrane GLUT4 protein did not increase above basal levels in either group. However, maximum insulin stimulation resulted in a significant increase in plasma-membrane GLUT4 protein concentration in both the NTG (4.1-fold) and TG (2.6-fold) mice. Despite the smaller increase in GLUT4 protein in response to maximal insulin stimulation, the TG mice maintained a significantly greater plasma-membrane GLUT4 protein concentration than the NTG mice ($\sim$ 8 times as high).

Under basal conditions, intracellular-membrane GLUT4 protein concentration was approximately four times higher in TG than in NTG mice. Submaximal insulin stimulation reduced intracellular-membrane GLUT4 protein concentration by 23 % in TG mice and 46 % in NTG mice. Maximal insulin stimulation reduced intracellular-membrane GLUT4 protein by 27 % and 58 % in TG and NTG mice respectively. Under all conditions the intracellular-membrane GLUT4 protein concentration was significantly higher in TG mice than in NTG mice.

DISCUSSION

The present study was designed to characterize the effects of GLUT4 protein overexpression on GLUT4 protein subcellular distribution and its relationship to glucose transport and metab-

Figure 2   Plasma- and intracellular-membrane GLUT4 protein concentration prepared from TG and NTG mouse skeletal muscle

The numbers of TG and NTG mice used are the same as in Table 2. Values are expressed as a percentage of a rat microsomal membrane standard and presented as means ± S.E.M. ■, Basal; □, submaximal insulin; ⊠, maximal insulin. *Significantly different from basal ($P < 0.05$); †significantly different from NTG ($P < 0.05$).

olism in skeletal muscle. In order to examine the translocation of GLUT4 protein in TG mouse skeletal muscle, it was necessary to develop a membrane-fractionation procedure for small muscle volumes. To our knowledge, only one other procedure for preparation of membranes from mouse skeletal muscle has been published [22]. However, this procedure did not produce membrane protein yields or fold purities that were similar to previous values from rat muscle. Consequently, in the present study we chose to modify the procedure of Hirshman et al. [6] as outlined in the Results section. We have previously characterized rat muscle membrane fractions isolated via this procedure by Western blotting for $(Na^+ + K^+)$-ATPase (plasma-membrane marker) and DHP receptors (T-tubule marker), and found that the plasma membrane was enriched in $(Na^+ + K^+)$-ATPase and the intracellular membrane fraction was enriched in DHP receptors. To determine if the modified membrane-isolation procedure produced membranes from mouse muscle that were similar to those previously isolated from rat muscle, we measured the plasma-membrane marker enzyme KpNPPase as well as Western blotting the membrane fractions for $(Na^+ + K^+)$-ATPase and DHP receptor. The mouse muscle membranes isolated by the modified procedure had KpNPPase activities and fold purities that were identical with those obtained from large preparations of rat muscle despite having lower yields of this membrane marker. In addition, comparison of Western blotting of mouse membranes from the present study and rat membranes showed virtually identical marker protein distribution with respect to GLUT4, DHP receptors and $(Na^+ + K^+)$-ATPase. Therefore the present procedure produces membranes that are of composition identical with those previously obtained from rat muscle.

Although some previous studies have indicated that GLUT4 protein may be translocated to the T-tubules on insulin stimulation, as well as the plasma membrane, the membrane marker distribution and insulin-stimulated GLUT4 translocation in membranes from the present study are identical with those from previous studies [6,19]. Therefore, although the enrichment of the intracellular membrane with DHP receptors may compromise the amount of GLUT4 protein translocation that was observed, the present results are representative of other published membrane-isolation studies [5,6,10,23]. Nevertheless, the results of the present study should be considered with this caveat in mind.

Measurement of GLUT4 protein in isolated plasma membranes under basal conditions indicated that the concentration of glucose transporters in TG mice was approx. 12 times that in NTG mice. These results are in agreement with those of Treadway et al. [15] in which immunocytochemical localization of GLUT4 protein in quadriceps muscle from TG mice indicated a marked increase in plasma-membrane-associated GLUT4 protein in the basal state. Furthermore, evaluation of the intracellular membranes under basal conditions revealed that TG mice had approximately four times higher GLUT4 protein concentration than NTG mice. In response to a submaximal insulin concentration, there was no increase in plasma-membrane GLUT4 protein concentration in either the NTG or TG mice, although there was a significant decline in their intracellular-membrane GLUT4 protein concentration. This could indicate that the membrane-isolation procedure used in the present study is not sensitive enough to detect a small increase in plasma-membrane GLUT4 or that stimulation by a submaximal concentration of insulin causes movement of GLUT4 to an intracellular compartment that is not recovered in the isolation procedure. In response to maximal insulin stimulation, both TG and NTG mice displayed significant increases in plasma-membrane and decreases in intracellular-membrane GLUT4 protein. The NTG mice exhibited a larger increase in plasma-membrane GLUT4 protein above basal (4.1-fold) than the TG mice (2.6-fold). Likewise, the decrease in intracellular-membrane GLUT4 protein was approximately 58 % from basal in the NTG mice and only 30 % in the TG mice. These findings suggest that overexpression of the GLUT4 protein 'overloaded' the vesicular targeting pathway or saturated the intracellular GLUT4 compartment [13,24] resulting in a substantially greater number of transporters associated with the plasma membrane, and reducing the percentage of insulin-stimulated transporter translocation in the TG mice.

Although there was a substantial difference in plasma-membrane GLUT4 protein concentration between muscle of TG and NTG mice under basal conditions, only a small (18 %) statistically insignificant increase in the rate of glucose transport was observed in this study. These findings are slightly different from the results of Treadway et al. [15] who found a 25 % increase in basal glucose transport in soleus muscle from TG mice compared with NTG mice. However, in the present study the difference in basal glucose transport in the muscle of TG and NTG mice was only slightly less than in the study of Treadway et al. [15]. Thus, with additional observations, we also may have found a significant difference in basal glucose transport between muscle of TG and NTG mice. It is also possible that the difference between the two studies reflects the difference in muscle fibre types studied. Nevertheless, both studies clearly demonstrate that basal muscle glucose transport is much lower in TG mice than would be predicted from their plasma-membrane GLUT4 protein concentration. In contrast with the results on skeletal muscle, Shepherd et al. [24] found that a 6–9-fold overexpression of GLUT4 protein in adipocytes resulted in a 20–34-fold increase in basal glucose transport. The reason for this discrepancy is not known, but could be due to differences in membrane composition or GLUT4 protein regulatory control between adipocytes and skeletal muscle.

It is of interest to note that exercise training, which induces a degree of GLUT4 protein overexpression, results in a 2–3-fold increase in plasma-membrane GLUT4 protein concentration in rat skeletal muscle, but causes little or no increase in glucose transport in the basal state [23]. This finding and those of the current study suggest that a large number of GLUT4 transporters can be associated with the plasma membrane in the basal state

while remaining inactive. In support of this interpretation, it was reported by Vannucci et al. [25] that GLUT4 protein can exist in two states at or near the adipocyte plasma membrane: a functional form and an 'occluded' or non-functional form not accessible to the extracellular milieu.

Although no increase in plasma-membrane GLUT4 protein concentration was detected after submaximal insulin stimulation, glucose transport was significantly increased in muscle of both the TG and NTG mice. The increased rate of transport for the NTG mice was approx. 2.5-fold above basal whereas in TG mice the increase was approx. 4.4-fold above basal. A possible explanation for this disparity is that the technique used to assess plasma-membrane GLUT4 protein is not sensitive enough to detect small but significant increases in plasma-membrane GLUT4 protein. Conversely, the increase in glucose transport without a concomitant increase in plasma-membrane GLUT4 protein could reflect exposure of transporters, already associated with the plasma membrane, to the exofacial surface.

In response to maximal insulin stimulation, muscle glucose transport was increased above basal approx. 7.5-fold in NTG mice and approx. 11.5-fold in TG mice. Although the muscle of the TG mice had plasma-membrane GLUT4 protein concentrations that were 8–12 times higher than those of the NTG mice, the differences in the rates of glucose transport between the TG and NTG mice under maximal insulin stimulation were quite modest (55 %). This is supported by the results of Shepherd et al. [24] in which they found that adipocytes from TG mice that overexpress the GLUT4 protein 6–9-fold displayed only a 2–4-fold greater insulin-stimulated glucose transport than adipocytes from NTG mice. These findings suggest that there is a limit to the number of GLUT4 transporters that can be exposed to the plasma-membrane surface and/or activated. Alternatively, it is possible that the disparity between plasma-membrane GLUT4 protein concentration and glucose transport is due to cross-contamination of the plasma-membrane fraction with intra-cellular membranes also containing the GLUT4 protein, thus inflating the levels of GLUT4 protein in the plasma membrane and reducing the apparent response to insulin.

Despite a 55 % difference in maximal glucose transport, the rate of muscle glucose metabolism was the same for the TG and NTG mice when perfused with a maximal insulin concentration. The rates of [^{14}C]glucose incorporation into glycogen, lactate accumulation and glucose oxidation for the TG and NTG mice were all similar. This suggests that intracellular glucose disposal was rate-limiting during maximal insulin stimulation in the TG mice. This is in agreement with the findings of Kubo and Foley [26] who found that, for perfusate glucose concentrations above 2 mM, glucose disposal and not transport was rate-limiting during hind-limb perfusion in rats. A possible limiting factor to glucose disposal is the activity of hexokinase. In the present study, muscle hexokinase activities in TG mice were 17 % lower than those in NTG mice. This decrement in hexokinase activity therefore could have contributed to the lack of difference in maximal insulin-stimulated glucose metabolism between the NTG and TG mice.

Recently, Hansen et al. [27] reported elevated rates of maximal-insulin-stimulated glycolytic flux in GLUT4 TG mice, which does not agree with the results of the present investigation. However, the rates of insulin-stimulated glycolytic utilization in the study of Hansen et al. [27] (10.12 μmol of glucose/h per g wet wt. for NTG mice compared with 21.1 μmol of glucose/h per g wet wt. for TG mice) are significantly higher than those of the present study (8.9 compared with 11.3 μmol of glucose/h per g wet wt.). In addition, the ratio of glycolytic flux to glycogen synthesis is approximately 4–5-fold higher in the study of Hansen

et al. [27] than in the present study. Thus it appears that the isolated extensor digitorum longus muscles used in the study of Hansen et al. [27] may have been stimulated by contraction or hypoxia, in addition to insulin. The increase in glycolytic flux could reduce the accumulation of glucose 6-phosphate in the presence of maximal insulin stimulation and prevent hexokinase from becoming rate-limiting as predicted in the present study. In the present study rates of glycolytic flux and glycogen synthesis were determined during hind-limb perfusion, which circumvents problems with tissue perfusion and diffusion, and stimulation of muscle by contraction during isolation and removal.

In summary, it was found that overexpression of an 11.5 kb human GLUT4 gene in mouse skeletal muscle resulted in a significant elevation in plasma- and intracellular-membrane GLUT4 protein concentration. Muscle glucose-transport rates during submaximal and maximal insulin stimulation were greater in TG than NTG mice, but glucose transport in TG mice during both basal and insulin-stimulated states was considerably less than would be predicted from plasma-membrane GLUT4 protein levels. Therefore these results suggest that factors in addition to plasma-membrane GLUT4 protein concentration regulate glu-cose transport. Furthermore, our results indicate that, during maximal insulin stimulation, the metabolic pathways of glucose metabolism are maximally active and incapable of further activation to accommodate the increase in glucose transport following overexpression of GLUT4 protein. Hence, under the experimental conditions imposed, glucose metabolism and not glucose transport is rate-limiting.

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