Use of a Novel Impermeable Biotinylated Photolabeling Reagent to Assess Insulin- and Hypoxia-Stimulated Cell Surface GLUT4 Content in Skeletal Muscle From Type 2 Diabetic Patients

Jeffrey W. Ryder, Jing Yang, Dana Galuska, Jorge Rincón, Marie Björnholm, Anna Krook, Sten Lund, Oluf Pedersen, Harriet Wallberg-Henriksson, Juleen R. Zierath, and Geoffrey D. Holman

Cell surface GLUT4 levels in skeletal muscle from nine type 2 diabetic subjects and nine healthy control subjects have been assessed by a new technique that involves the use of a biotinylated photo-affinity label. A profound impairment in GLUT4 translocation to the skeletal muscle cell surface in response to insulin was observed in type 2 diabetic patients. Levels of insulin-stimulated cell surface GLUT4 above basal in type 2 diabetic patients were only ~10% of those observed in healthy subjects. The magnitude of the defect in GLUT4 translocation in type 2 diabetic patients was greater than that observed for glucose transport activity, which was ~50% of that in healthy subjects. Reduced GLUT4 translocation is therefore a major contributor to the impaired glucose transport activity in skeletal muscle from type 2 diabetic subjects. When a marked impairment in GLUT4 translocation occurs, the contribution of other transporters to transport activity becomes apparent. In response to hypoxia, marked reductions in skeletal muscle cell surface GLUT4 levels were also observed in type 2 diabetic patients. Therefore, a defect in a common late stage in signal transduction and/or a direct impairment in the GLUT4 translocation process accounts for reduced glucose transport in type 2 diabetic patients. *Diabetes* 49:647–654, 2000

From the Department of Clinical Physiology, Karolinska Hospital, and the Department of Physiology and Pharmacology (J.W.R., D.G., J.R., M.B., A.K., H.W.-H., J.R.Z.), Karolinska Institute, Stockholm, Sweden; the Department of Biochemistry (J.Y., G.D.H.), University of Bath, Claverton Down, Bath, U.K.; the Medical Research Laboratory and the Medical Department M (S.L.), Kommunehospitalet, Aarhus University Hospital, Aarhus; and the Steno Diabetes Center and Hagedorn Research Institute (O.P.), Copenhagen, Denmark.

Address correspondence and reprint requests to Juleen R. Zierath, PhD, Associate Professor of Physiology, Department of Clinical Physiology, Gustaf V's Research Institute, Karolinska Hospital, S-171 76 Stockholm, Sweden. E-mail: jrz@klinfys.ks.se.

Received for publication 11 November 1999 and accepted 29 December 1999.

H.W.-H. and J.R.Z. have received honoraria for speaking engagements from Pharmacia and Upjohn.

AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride; ANOVA, analysis of variance; Bio-LC-ATB-BMPA, 4,4′-*O*-[2-[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; ECL, enhanced chemiluminescence; HES, HEPES EDTA sucrose; IRS, insulin receptor substrate; KHB, Krebs-Henseleit bicarbonate buffer; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; RIA, radioimmunoassay; TBS-T, Tris-buffered saline 0.1% Tween.

Skeletal muscle is the primary site of whole-body insulin-mediated glucose uptake (1). People with type 2 diabetes are characterized by reduced insulin-mediated whole-body glucose uptake (2), which appears to be a result of reduced insulin-stimulated glucose transport in skeletal muscle (3–7). Evidence suggests that reduced insulin-mediated glucose transport in skeletal muscle from type 2 diabetic patients results from alterations in the insulin-signal transduction pathway (8–14). We have shown that an increase in fasting serum insulin levels (~50 to ~600 pmol/l), achieved by a hyperinsulinemic clamp, increased insulin signal transduction at the level of insulin receptor substrate (IRS)-1 and phosphatidylinositol 3-kinase (PI3K) (13) and promoted GLUT4 translocation from an intracellular storage site to the plasma membrane in skeletal muscle from healthy individuals (15,16). Conversely, in type 2 diabetic patients, insulin signal transduction (13) and plasma membrane GLUT4 content in skeletal muscle (16) were markedly reduced.

Whether reduced insulin-stimulated glucose transport occurs as a consequence of a defect in the mechanisms involved in GLUT4 traffic in skeletal muscle from type 2 diabetic patients or as a consequence of impaired insulin signal transduction remains to be determined. Alterations in the traffic and/or translocation of GLUT4 to the plasma membrane have previously been proposed to contribute to the reduced insulin-stimulated glucose uptake in skeletal muscle from type 2 diabetic and nondiabetic insulin-resistant individuals (6,16–18). A recent analysis of the subcellular distribution of GLUT4 in the basal (noninsulin-stimulated) state provides evidence to suggest that defects in GLUT4 trafficking and translocation are a cause of insulin resistance in skeletal muscle (18). Thus, in addition to insulin-signaling defects, insulin resistance may also be due to a failure of GLUT4 vesicles to translocate, dock, or fully fuse with the plasma membrane.

A means of addressing the question of whether a trafficking impairment contributes to the insulin resistance in type 2 diabetes is to examine the translocation of GLUT4 in response to stimuli other than insulin. In skeletal muscle, glucose transport can be activated in response to both insulin and muscle contraction and/or exercise (5,19–21). There is strong evidence for a role for PI3K in insulin-stimulated but not con-

traction-stimulated glucose transport and GLUT4 translocation (19–21). Activation of 5′-AMP–activated kinase (22,23) and increases in cytoplasmic calcium levels (24,25) may be involved in the contraction response, because they both lead to insulin-independent increases in glucose transport. Exposure of isolated skeletal muscle to hypoxia also leads to an insulin-independent increase in glucose transport and GLUT4 translocation (26–28). Hypoxia and exercise are believed to increase transport by a similar mechanism (26). Therefore, perturbing the muscle contraction–hypoxia pathway is a strategy to reveal whether the insulin resistance associated with type 2 diabetes is limited to the insulin-signaling cascade or is a consequence of generalized resistance in the mechanism(s) involved in GLUT4 translocation.

Questions as to whether defects in GLUT4 translocation occur in type 2 diabetes have been a challenge to address in human skeletal muscle, because the most commonly used analytical methods to assess GLUT4 traffic require large amounts of material (up to 1 g/perturbation) for subcellular membrane fractionation procedures. These methods are used to obtain separate purified plasma membranes and low-density microsomes (15–18,29,30). Bis-mannose photolabels (31) are now available to quantify the magnitude of the insulin response on GLUT4 translocation in skeletal muscle (32). We have previously (33) used radioactively labeled probes to estimate the cell surface GLUT4 content in skeletal muscle from healthy subjects. Recently, sensitive nonradioactive biotinylated bis-mannose photolabels have been described (34). Methods that use these reagents take advantage of the strong interaction with the biotin-binding protein, streptavidin, and provide a means to detect GLUT4 in small samples (20 mg) of human skeletal muscle. Here, we use the new bis-mannose photolabeling technique to determine whether decreased insulin-stimulated glucose transport in skeletal muscle from type 2 diabetic patients is associated with a decreased abundance of GLUT4 in the plasma membrane. Secondly, we incubated skeletal muscle under conditions of hypoxia to assess whether the insulin resistance associated with type 2 diabetes is specific to the insulin-mediated pathway or general to the processes mediating GLUT4 translocation.

RESEARCH DESIGN AND METHODS

Subjects. The Institutional Ethical Committee of the Karolinska Institute approved the study protocol. Informed consent was received from all subjects before participation. Clinical characteristics of the study participants are presented in Table 1. A total of nine type 2 diabetic men with a mean time since diagnosis of disease of 6 ± 1 years (range 2–15) were studied. Glycemic control, as evaluated by HbA_{1c} concentration, was moderate (6.4 ± 0.5%). Normal values for HbA_{1c} in our laboratory are <5.2%. Of the nine subjects, three were treated with either insulin, diet, or a combined treatment of acarbose and sulfonylureas, and six were treated with sulfonylureas alone. The control group consisted of nine healthy men. None of the study participants smoked. All subjects underwent a physical examination and were not taking any other medications known to alter carbohydrate metabolism. The subjects were instructed to abstain from any form of strenuous physical activity for a period of 48 h before the experiment and to maintain their normal diet. The subjects reported to the laboratory after an overnight fast and, in the case of the type 2 diabetic patients, before administration of any antidiabetic medication. In vitro and in vivo assessments of insulin action were performed on separate occasions separated by 4–8 weeks.

Euglycemic-hyperinsulinemic clamp studies. Whole-body insulin-mediated glucose uptake was determined by the euglycemic-hyperinsulinemic clamp technique (35). A catheter was inserted into an anticubital vein for glucose and insulin infusion and into the brachial artery for blood sampling. After collection of baseline samples, a bolus dose of insulin was infused (24 pmol · kg^{-1} · min^{-1} for 2 min, 12pmol · kg^{-1} · min^{-1} for 6 min). Thereafter, insulin was administered by continuous infusion at a rate of 6 pmol · kg^{-1} · min^{-1}. The plasma glucose concentration (~5 mmol/l) was kept constant for 100 min by a variable glucose infusion that was adjusted after plasma glucose measurements every 5 min (glucose oxidase method). Blood samples for insulin and glucose steady-state determinations were collected at 30-min intervals. Fasting glucose and insulin levels at steady state were 4.70 ± 0.08 mmol/l and 682 ± 11 pmol/l for the control subjects and 4.74 ± 0.04 mmol/l and 677 ± 12 pmol/l for the type 2 diabetic subjects, respectively (NS). Whole-body glucose uptake was calculated from the glucose infusion rate (μmol · kg^{-1} · min^{-1}) required to maintain steady state.

Blood chemistry. Plasma glucose levels were measured by a glucose-oxidase method. Serum immunoreactive insulin was assayed by the Phadeseph Insulin Radioimmunoassay (RIA) method (Pharmacia, Uppsala, Sweden). The lower limit of sensitivity of this method is 18 pmol/l of insulin. HbA_{1c} values were determined by specific ion-exchange chromatography, using a kit (mono S HR 5/5; Pharmacia). Plasma free fatty acid levels were determined using a microfluorometric method (36). Serum triglyceride, HDL cholesterol, and LDL cholesterol levels were assessed by reflectance spectrometry by use of Kodak Ektachem Clinical Chemistry Slides (Eastman Kodak, Rochester, NY).

Maximal oxygen uptake determination. On a separate occasion, Vo_{2max} was determined on a bicycle ergometer as described (37). Vo_{2max} was measured continuously with a breath-by-breath data collection technique (Erich Jaeger, Hoechberg, Germany) and calculated at each 20-s interval.

Muscle biopsy studies. Glucose transport was determined by an in vitro method described for human skeletal muscle used routinely in our laboratory (4,5,13,14,16). A muscle biopsy (1 g) was obtained under local anesthesia (Mepivakain chloride 5 mg/ml) from the vastus lateralis portion of the quadriceps femoris, as previously described (5). Muscle specimens (20 mg) were dissected from the biopsy material, mounted on Plexiglas clips (9 mm in width), and placed in individual flasks containing oxygenated Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 5 mmol/l HEPES, 18 mmol/l mannitol, 2 mmol/l pyruvate, and 0.1% bovine serum albumin (RIA Grade; Sigma, St. Louis, MO). The incubation flasks were placed in a shaking water bath (60 times/min) with a constant temperature (35°C) and continuous oxygenation (95% O_2/5% CO_2).

Muscle incubation procedure. Muscles were transferred to KHB media containing 5 mmol/l glucose and pre-incubated for 60 min in the absence or presence of insulin (120 nmol/l). To examine the effects of hypoxia on glucose transport and cell surface GLUT4 content, muscles were pre-incubated for 60 min, as described above, in KHB containing 5 mmol/l glucose and 15 mmol/l mannitol, with or without insulin (120 nmol/l), under a gas phase of 95% N_2/5% CO_2 (hypoxia). After the pre-incubation, muscle samples were rinsed (10 min) in oxygenated glucose-free media (35°C) with continuous oxygenation (95% O_2/5% CO_2).

Glucose transport measurements. Muscles were transferred to media containing 5 mmol/l [^{3}H]-3-*O*-methylglucose (800 μCi × $mmol^{-1}$) and 15 mmol/l [^{14}C]mannitol (53 μCi × $mmol^{-1}$) and incubated for 20 min. Glucose transport was evaluated by measuring the accumulation of [^{3}H]-3-*O*-methylglucose and [^{14}C]mannitol as described by Wallberg-Henriksson et al. (38). Glucose transport activity is expressed as micromoles of glucose analog accumulated per milliliter of intracellular water per hour.

TABLE 1
Clinical characteristics of the study participants

	Control subjects	Type 2 diabetic subjects
n	9	9
Age (years)	55 ± 1	57 ± 2
BMI (kg/m^2)	24.9 ± 0.5	26.4 ± 0.6
Fasting blood glucose (mmol/l)	4.8 ± 0.2	8.5 ± 0.8*
Fasting serum insulin (pmol/l)	35 ± 4	96 ± 21†
HbA_{1c} (%)	4.7 ± 0.1	6.4 ± 0.5‡
Free fatty acid (μmol/l)	446 ± 62	425 ± 35
Triglycerides (mmol/l)	1.2 ± 0.3	1.8 ± 0.2
Cholesterol (mmol/l)	5.4 ± 0.5	5.4 ± 0.4
HDL cholesterol (mmol/l)	1.3 ± 0.8	1.2 ± 0.1
LDL cholesterol (mmol/l)	3.4 ± 0.5	3.3 ± 0.3
Insulin-mediated glucose uptake (μmol · kg^{-1} · min^{-1})	41.8 ± 2.1	27.1 ± 4.0†
Vo_{2max} (ml · kg^{-1} · min^{-1})	33.8 ± 3.2	33.1 ± 3.7

Data are means ± SE. *P < 0.001, †P < 0.01, and ‡P < 0.005 vs. control subjects.

Cell surface GLUT4-labeling procedure. After the initial pre-exposure to insulin and/or hypoxia, as previously described, muscles were rinsed (10 min) in oxygenated glucose-free KHB media (18°C) and then incubated for 8 min in KHB containing 400 µmol/l Bio-LC-ATB-BMPA (4,4′-*O*-[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). Muscles were then irradiated twice for 3 min in a Rayonet photochemical reactor (Southern New England Ultraviolet, Branford, CT) using 300-nm lamps. The muscles were frozen and stored at –80°C until the time of analysis. Approximately 15–20 mg wet weight of labeled muscle per condition was homogenized (30 strokes at 0–4°C on ice) in 400 µl HEPES EDTA sucrose (HES) buffer (255 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l HEPES, 1 µg/ml antipain, aprotinin, pepstatin, leupeptin, and 100 µmol/l 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride [AEBSF], pH 7.2). Homogenates were washed once with 400 µl of HES buffer and subjected to centrifugation (227,000*g* for 50 min at 4°C) to obtain a total membrane fraction. This pellet was solubilized in 0.5 ml of phosphate-buffered saline (PBS), pH 7.2, 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 µmol/l AEBSF). The samples were solubilized for 50 min at 4°C with rotation and were then subjected to centrifugation (20,000*g* for 20 min at 4°C). Biotinylated proteins in the supernatants were precipitated with 50 µl of streptavidin beads (Pierce, Rockford, IL). The precipitates were washed four times with 1 ml of PBS buffer containing 1% Thesit with protease inhibitors, four times with 1 ml PBS containing 0.1% Thesit plus protease inhibitors, and once in 1 ml of PBS. Thereafter, 35 µl of electrophoresis sample buffer (62.5 mmol/l Tris, pH 6.8, 2% SDS, 10% glycerol) was added to each pellet. The sample was then heated to 95°C for 30 min. The samples were subjected to centrifugation (2,300*g* for 1 min), and the supernatants were removed. The pellets were washed with 30 µl of electrophoresis sample buffer, heated to 95°C for 30 min, and resubjected to centrifugation. Supernatant fractions were pooled, mercaptoethanol was added to 10% of the samples, and they were then subjected to SDS-PAGE (10% gel). Proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline 0.1% Tween (TBS-T) and were washed six times with TBS-T. Membranes were incubated with affinity-purified anti-GLUT4 COOH-terminal antibody (31) in TBS-T containing 1% bovine serum albumin (2 h at room temperature), followed by washing (six times in TBS-T) and detection by using a secondary antibody linked to horseradish peroxidase. GLUT4 protein was visualized with enhanced chemiluminescence (ECL). GLUT4 levels were estimated by comparison with a series of dilutions of a standard rat adipocyte low-density microsome membrane fraction containing 60 pmol/mg of GLUT4 (39). Multiple film exposures were used to ensure that GLUT4 was detected in the linear range of the standards.

Total expression of GLUT4 and GLUT1 protein. Crude membranes (plasma membrane and microsome) were prepared as described (40). Muscle membranes were subjected to SDS-PAGE, followed by immunoblot analysis. GLUT4 expression was determined and quantitated as previously described. GLUT1 expression was determined by immunoblotting with a polyclonal anti–COOH-terminal peptide antibody (Diagnostic International, Karlsdorf, Germany), and levels were estimated with a series of dilutions of human erythrocyte ghosts containing 500 pmol/mg of GLUT1, as determined by cytochalasin B binding (41). Immunolabeled bands were visualized by ECL, and autoradiograms were quantitated by scanning densitometry.

Statistics. Results are presented as means ± SE. Paired Student's *t* tests were used for statistical analysis of stimulatory responses in glucose transport and GLUT4 labeling. Unpaired Student's *t* tests were used for statistical analysis of the subject characteristics and for analysis of the glucose transport or GLUT4-labeling differences between control and type 2 diabetic subjects. The relationship between insulin-stimulated glucose transport and cell surface GLUT4 was determined by use of Spearman's correlation coefficient. Two-way analysis of variance (ANOVA) was used to compare response differences as measured by transport activity or cell surface GLUT4. $P < 0.05$ was considered to be statistically significant.

RESULTS

Subject characteristics. Subjects were normal to moderately overweight and were matched for age, BMI, and physical fitness (Table 1). Thus, any physiological difference between the type 2 diabetic and control subjects is not likely to be explained by either poor fitness (reduced Vo_{2max}) or obesity (BMI >30 kg/m^2). Fasting blood glucose levels and serum insulin levels were significantly elevated in the type 2 diabetic subjects (Table 1). However, the blood lipid profiles were similar between type 2 diabetic and control subjects. HbA_{1c} levels were moderately elevated, suggesting the type 2 diabetic subjects were in relatively good metabolic control. Whole-body insulin-mediated glucose uptake was significantly impaired in the type 2 diabetic subjects (36% reduction compared with that of control subjects, $P < 0.01$).

Glucose transport activity. Isolated skeletal muscle from control or type 2 diabetic subjects was incubated in the absence or presence of insulin (120 nmol/l), and glucose transport was assessed (Fig. 1). Basal glucose transport was similar between type 2 diabetic and control subjects. In control subjects, insulin elicited a 4.3 ± 0.6–fold increase in 3-*O*-methylglucose transport activity ($P < 0.001$). In type 2 diabetic subjects, though, insulin elicited a 2.3 ± 0.2–fold increase in 3-*O*-methylglucose transport activity ($P < 0.01$). However, insulin-stimulated glucose transport activity was reduced by 40% ($P < 0.03$) in skeletal muscle from type 2 diabetic compared with that from control subjects.

To determine whether the reduced insulin-stimulated glucose transport was due to a defect in the insulin-stimulated pathways for glucose transport or general resistance of GLUT4 vesicle traffic, skeletal muscle from control or type 2 diabetic subjects was incubated under conditions of hypoxia for 60 min, and glucose transport activity was assessed. In vitro exposure to hypoxia led to a 3.6 ± 0.6–fold increase in 3-*O*-methylglucose transport activity ($P < 0.001$) in control

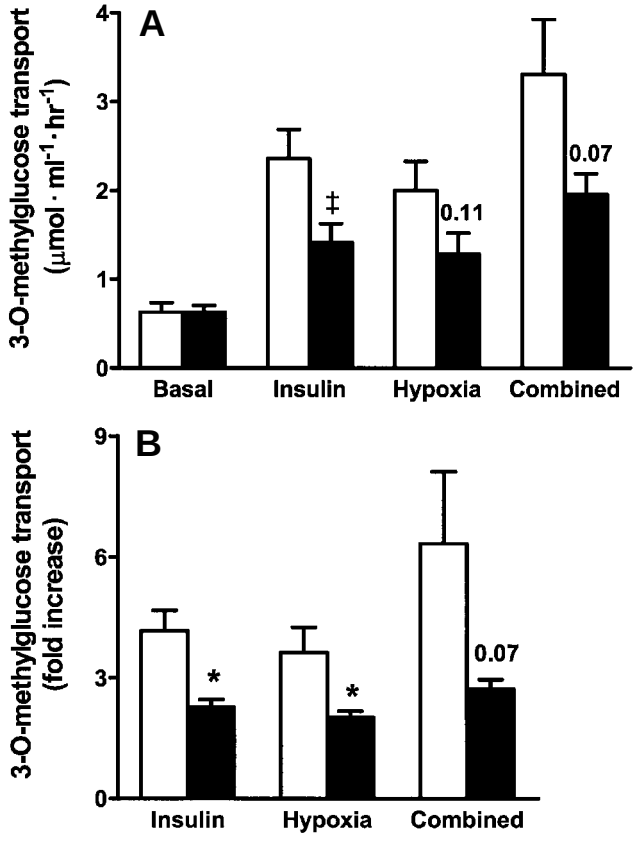

FIG. 1. Insulin- and hypoxia-stimulated 3-*O*-methylglucose transport in isolated skeletal muscle from control and type 2 diabetic subjects. Glucose transport was expressed as micromoles of 3-*O*-methylglucose per milliliter of intracellular water per hour (*A*), or as fold over basal (noninsulin-stimulated condition) (*B*) for control (□) and type 2 diabetic subjects (■). Data are means ± SE (n = 9 for basal, insulin, and hypoxia, and n = 6 for insulin plus hypoxia in control subjects; n = 9 for basal and insulin, 7 for hypoxia, and 6 for insulin plus hypoxia in type 2 diabetic subjects). *$P < 0.05$ and ‡$P < 0.005$ or actual P value vs. the same condition in control subjects.

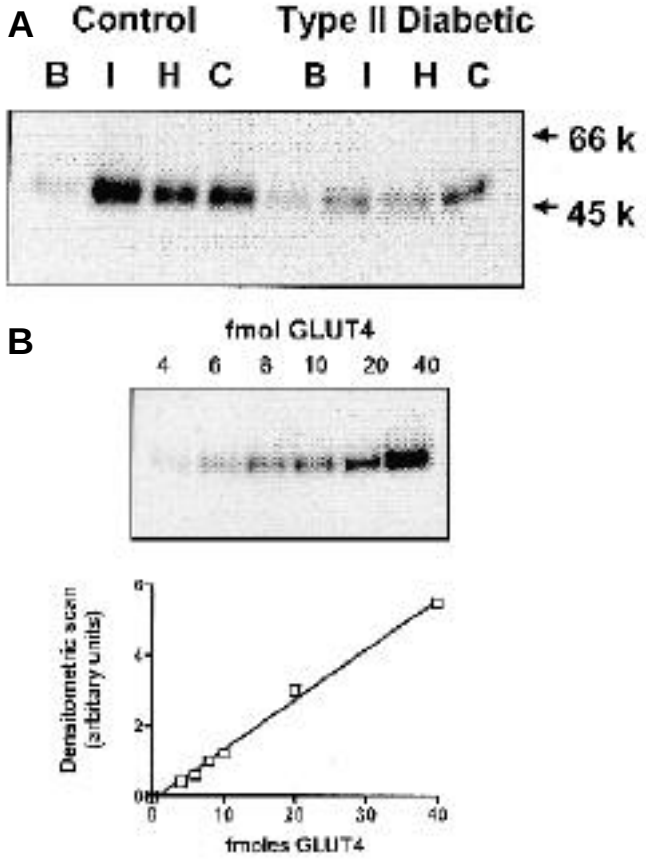

FIG. 2. Detection of biotinylated GLUT4 at the cell surface of isolated skeletal muscle from control and type 2 diabetic subjects. *A*: After labeling of skeletal muscle with biotinylated photolabel, streptavidin-agarose precipitation was used to isolate the cell surface GLUT4. This was subsequently resolved on SDS-PAGE and detected with an anti–COOH-terminal GLUT4 antibody. Levels of cell surface GLUT4 in control (left panels) and type 2 diabetic subjects (right panels) were compared in basal- (B), insulin- (I), hypoxia- (H), and combined insulin- and hypoxia (C)-stimulated muscle. *B*: To quantify these levels of GLUT4 in human muscle, the immunoreactive signals were compared with a series of dilutions of a rat adipocyte membrane standard in which the levels of GLUT4 were quantified from cytochalasin B–binding data. The data shown are from typical analyses.

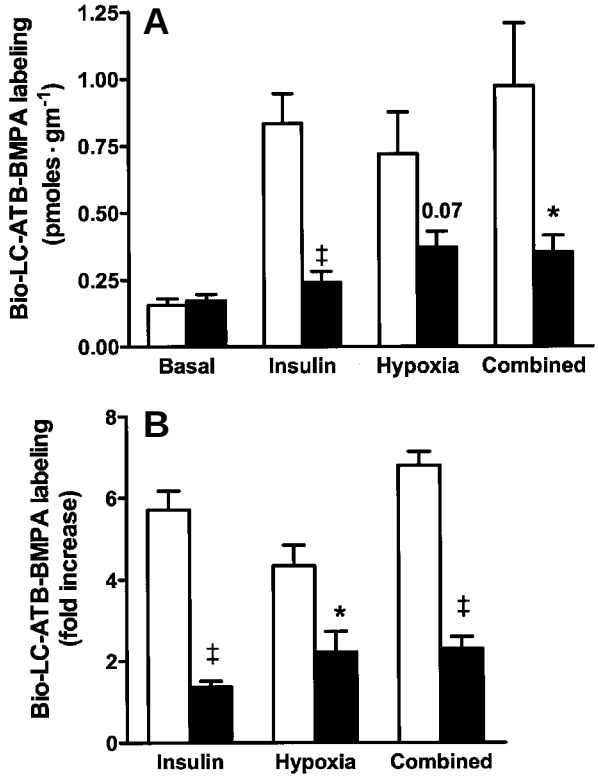

FIG. 3. Insulin- and hypoxia-stimulated cell surface GLUT4 labeling in isolated skeletal muscle from control and type 2 diabetic subjects. The cell surface GLUT4 content is expressed as the cell surface amount, picomoles of GLUT4 per gram of wet weight muscle (*A*), or as fold over basal (noninsulin-stimulated condition) (*B*) for control (□) and type 2 diabetic subjects (■). Data are means ± SE, and *n* is reported in the Fig. 1 legend. **P* < 0.05 and ‡*P* < 0.005 or actual *P* value vs. the same condition in control subjects.

subjects, and this increase was comparable to the effect achieved by insulin-stimulation. Hypoxia resulted in a 2.0 ± 0.2–fold increase in glucose transport activity in type 2 diabetic subjects ($P < 0.01$), and, as noted in the control subjects, this increase was comparable to the effect achieved by insulin-stimulation. Hypoxia-mediated glucose transport activity was 44% lower ($P < 0.05$) in type 2 diabetic subjects compared with that of control subjects (Fig. 1*B*).

In control subjects, the combined effect of a maximal insulin stimulus and hypoxia were partly additive (1.5- and 1.7-fold increase compared with the effect achieved by insulin or hypoxia alone). However, these differences were not statistically greater than the response achieved by insulin or hypoxia alone. In skeletal muscle from type 2 diabetic subjects, the combined effect of a maximal insulin stimulus and hypoxia on 3-*O*-methylglucose transport was not significantly different from that of either stimulus alone.

Cell surface GLUT4 levels in skeletal muscle. Skeletal muscle was incubated in the absence or presence of insulin (120 nmol/l) or under conditions of hypoxia with or without insulin, as described in RESEARCH DESIGN AND METHODS. A typical scan of cell surface GLUT4 content in skeletal muscle from control or type 2 diabetic subjects is shown in Fig. 2*A*. A typical standard curve of a series of dilutions of a rat adipocyte GLUT4 standard, as shown in Fig. 2*B*, was used to estimate GLUT4 content in pmol/g wet weight of muscle. The assay was linear over the range of test samples and GLUT4 standards.

Basal levels of cell surface GLUT4 were similar between control and type 2 diabetic subjects (Fig. 3*A*). In control subjects, insulin elicited a 5.7 ± 0.5–fold increase in cell surface GLUT4 levels ($P < 0.001$ vs. basal). In type 2 diabetic subjects, though, insulin elicited a 1.4 ± 0.1–fold increase in cell surface GLUT4 content ($P < 0.02$). Thus, insulin-stimulated cell surface GLUT4 content was 71% lower ($P < 0.001$) in muscle from type 2 diabetic subjects compared with that from control subjects (Fig. 3*B*).

We next assessed the effect of hypoxia on cell surface GLUT4 levels in skeletal muscle (Fig. 3). In control subjects, cell surface GLUT4 content was increased 4.3 ± 0.5–fold ($P < 0.001$) in response to hypoxia alone and 6.8 ± 0.3–fold ($P < 0.05$) in response to co-incubation with a maximal insulin stimulus and hypoxia. This combined effect of a maximal insulin stimulus and hypoxia were partially, but insignificantly, additive (1.2- and 1.6-fold increase, respectively, compared with the effect of insulin or hypoxia alone). In skeletal muscle from type 2 diabetic subjects, cell surface GLUT4 content was

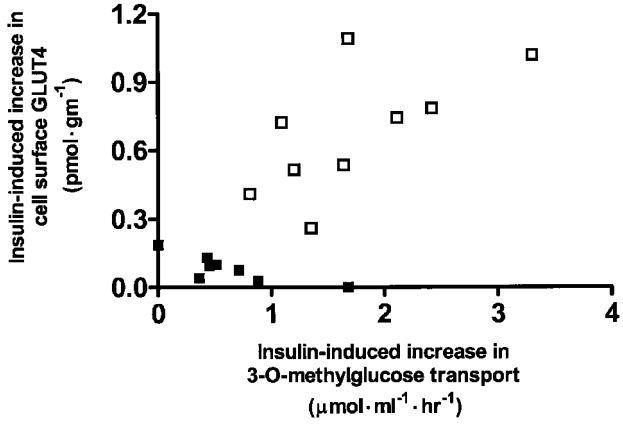

FIG. 4. Correlation between the insulin-induced increase in cell surface GLUT4 content and the insulin-induced increase in 3-*O*-methylglucose transport activity in skeletal muscle from control (□) and type 2 diabetic subjects (■). Insulin-stimulated minus basal data are presented. $r^2 = 0.62$ (Spearman's correlation); $P < 0.01$.

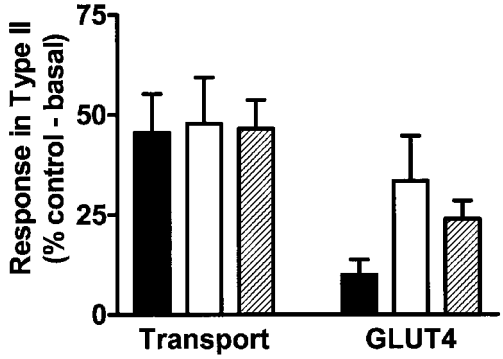

FIG. 5. Comparison of increases in cell surface GLUT4 and 3-*O*-methylglucose transport activity in type 2 diabetic subjects. The increases in glucose transport activity and cell surface GLUT4 in response to insulin (■), hypoxia (□), and combined insulin and hypoxia (▨) in type 2 diabetic subjects were calculated as a percentage of the corresponding average for the control subjects. All data sets were minus basal. In type 2 diabetic subjects, the attenuation of the normal control response was greater in GLUT4 translocation than in transport activity. Data are means ± SE, and *n* is reported in the Fig. 1 legend. $P < 0.005$ (two-way ANOVA).

increased 2.2 ± 0.5–fold ($P < 0.05$) in response to hypoxia. Thus, hypoxia-stimulated cell surface GLUT4 labeling was 49% lower in muscle from type 2 diabetic subjects compared with that from control subjects ($P < 0.01$). The combined effects of a maximal insulin stimulus and hypoxia on cell surface GLUT4 content in skeletal muscle from type 2 diabetic subjects were similar to the effects achieved by hypoxia alone, but they were slightly higher (1.7-fold) than the effects achieved with a maximal insulin stimulus ($P < 0.05$).

As reported in our earlier study of young healthy subjects (40), the insulin-induced increase in cell surface GLUT4 content was positively correlated with the insulin-mediated increase in 3-*O*-methylglucose transport ($r^2 = 0.62$, $P < 0.01$) (Fig. 4). Thus, the low GLUT4 content at the cell surface in type 2 diabetic subjects is largely responsible for the low insulin response in glucose transport activity. However, the correlation between cell surface GLUT4 content and glucose transport activity is not exact, and, in type 2 diabetic patients, where the cell surface GLUT4 content is low, there is relatively high residual glucose transport activity. This is also partially reflected in a comparison of the fold changes in glucose transport activity and cell surface GLUT4 content (Figs. 1*B* and 3*B*). Because the fold changes are relative to the basal values, a greater contribution of basal glucose transport activity in type 2 diabetic subjects could possibly contribute to these differences. However, when basal levels of glucose transport or cell surface GLUT4 content were subtracted and compared as a percentage of the mean insulin or hypoxia response in control subjects (Fig. 5), cell surface GLUT4 content was significantly lower than glucose transport activity in the type 2 diabetic patients ($P < 0.005$, two-way ANOVA).

Total skeletal muscle GLUT4 and GLUT1 levels. The total amount of GLUT4 and GLUT1 protein was compared between control and type 2 diabetic subjects. Comparison with a range of rat adipocyte low-density microsomes or human erythrocyte ghost membranes was used for the quantitation of GLUT4 and GLUT1, respectively. GLUT4 expression was similar between the control and type 2 diabetic subjects (4.13 ± 0.41 and 4.14 ± 0.32 pmol/g wet weight muscle for control and type 2 diabetic subjects, respectively). As previously reported (40), the presence of erythrocytes (which have extremely high levels of GLUT1 [500 pmol/mg membrane] [41]) in the muscle complicated the analysis of this GLUT isoform. However, in extensively washed muscle, GLUT1 content was ~1 pmol/g (~25% of the GLUT4 level). Nevertheless, because residual erythrocyte GLUT1 may still have been present in the washed muscle, the analysis of GLUT1 expression between control and type 2 diabetic subjects was not studied in detail.

DISCUSSION

In the present study, we exposed isolated skeletal muscle from type 2 diabetic patients to a maximal insulin stimulus and applied an exofacial photolabeling technique that uses the newly developed bis-mannose compound, Bio-LC-ATB-BMPA, to specifically determine the cell surface GLUT4 content. Because this label is impermeable, this technique detects only transporters that are present in the cell surface membrane. In isolated rodent soleus skeletal muscle, a six- to eightfold increase in 3-*O*-methylglucose transport after stimulation via the insulin (27,32,42) and/or the muscle contraction pathway (19) corresponds to a similar increase in cell surface GLUT4 content as assessed with the ATB-BMPA photolabel. Our initial study of human skeletal muscle confirms that insulin-stimulated glucose transport activity and cell surface GLUT4 content are closely associated (33). These results can be contrasted with the reported less-than-twofold insulin-stimulated increase in GLUT4 translocation observed by using subfractionation techniques on human skeletal muscle, where this methodology results in crossover between GLUT4 at the cell surface and contributions from GLUT4 in intracellular membranes (15–18,29,30). These crossover problems lead to an underestimate of the insulin effect on GLUT4 translocation. Consequently, resolving the extent to which this translocation is impaired in skeletal muscle from type 2 diabetic patients is difficult (16–18,30). Our new photolabel takes advantage of the strong interaction with the biotin-binding protein, streptavidin, allowing for quantitative determinations of cell surface GLUT4 levels in small samples of

human skeletal muscle (20 mg). With this more sensitive GLUT4 biotinylation technique, we have now been able to examine the insulin- and hypoxia-stimulated GLUT4 response in skeletal muscle from control and type 2 diabetic subjects. This approach has allowed the first detailed consideration of the contribution of GLUT4 to the impaired glucose transport activity in skeletal muscle from type 2 diabetic patients.

Our results show that type 2 diabetic patients have a markedly impaired response to both insulin and hypoxia, as revealed by the reduced exposure of GLUT4 at the skeletal muscle cell surface, versus that of control subjects. This finding suggests firstly that impaired GLUT4 translocation is a major contributor to the impaired glucose transport activity in type 2 diabetes. Surprisingly, we have found that in type 2 diabetic subjects, the degree of insulin resistance in the exposure of GLUT4 at the cell surface is greater than that observed for 3-*O*-methyl-D-glucose transport activity (Fig. 5). This suggests that when GLUT4 translocation is deficient, as in skeletal muscle from type 2 diabetic patients, there may be a relatively greater contribution of another transporter to the glucose transport activity. Analysis of GLUT1 showed a high level of expression (~25% of the GLUT4 level) in skeletal muscle, but the extent to which erythrocyte GLUT1 contributed to this level could not be determined in detail. Furthermore, the extent to which GLUT1 contributed to the glucose transport activity or the contribution of GLUT1 to the discrepancies observed between glucose transport and photolabeling could not be ascertained. An additional uncharacterized GLUT-like protein may possibly contribute to non-GLUT4–mediated glucose transport activity, as proposed for GLUT4 knockout mice (43,44). However, to date, there is no firm evidence for expression of additional functional glucose transporters in skeletal muscle.

Methodological differences in measuring 3-*O*-methylglucose transport and GLUT4 translocation may have contributed to the difference in the fold changes between these two parameters. For example, the subtraction of the non-transporter-mediated transport from the transporter-mediated transport or, alternatively, a background subtraction for the labeling experiments may contribute to the difference in the fold changes observed in the stimulatory responses to insulin and hypoxia. However, even after subtraction of the respective basal activity for glucose transport or cell surface GLUT4 content (Fig. 5), there is a marked reduction in the response to insulin in the type 2 diabetic patients. The insulin-induced stimulation above basal levels of GLUT4 labeling and glucose transport activity in type 2 diabetic subjects are only ~10 and ~50%, respectively, of those in the control subjects.

The marked impairment of GLUT4 translocation to the cell surface of skeletal muscle from type 2 diabetic patients has implications regarding whether impaired early signaling steps can fully account for this profound insulin resistance in glucose transport. Impairments in insulin-stimulated tyrosine phosphorylation of IRS-1 and in downstream signaling via PI3K have been observed in type 2 diabetic (14,45) or obese insulin-resistant subjects (12). The levels of insulin resistance seen in these early steps of the insulin-signaling pathway are generally moderate (<50%). In addition, there appears to be spare receptor and signaling capacity in the normal system that leads to GLUT4 translocation. The concentration of insulin required to activate glucose transport maximally corresponds to only 14% of the maximal receptor kinase activity (12,45). Insulin signaling may alter the cell surface GLUT4 levels by leading to a reduction in endocytosis or an increase in exocytosis (46,47). In rat adipocytes that are chronically treated with insulin, downregulation of cell surface GLUT4 content occurs because of an increase in endocytosis, and this process is more sensitive to relatively small (~50%) changes in the levels of early signaling intermediates (48). However, the profound reduction in GLUT4 translocation found in the present study (where the insulin response above basal in skeletal muscle from type 2 diabetic subjects is only ~10% of that from control subjects) is unlikely to result from a ninefold increase in the rate of endocytosis. It seems more likely that impairment(s) in the exocytosis components of GLUT4 translocation occur(s), and it is these steps that appear to require only low levels of stimulation of signaling (48). These considerations, therefore, suggest that insulin resistance in skeletal muscle may occur in the GLUT4-trafficking (probably exocytosis) process.

To further examine the possibility that there are impairments in the GLUT4 translocation process in skeletal muscle from type 2 diabetic patients that are independent of early insulin-signaling events, we have studied the translocation of GLUT4 in response to hypoxia. This stimulatory process does not appear to involve tyrosine phosphorylation of IRSs, activation of PI3K, or activation of Akt (19–21,26,49–51). In skeletal muscle from type 2 diabetic subjects, a marked impairment in GLUT4 labeling and glucose transport activity was noted in response to hypoxia. The implication from these results is that there is an impairment at or beyond a point of convergence of the insulin- and hypoxia-stimulatory pathways or that there is a resistant step directly at the level of GLUT4 translocation.

Because GLUT4 translocation is a complex process involving membrane vesicle trafficking and sorting machinery, there are many potential steps that may be impaired. The steps in the GLUT4 translocation process include budding or release of GLUT4 from an intracellular reservoir compartment, transit to the plasma membrane (possibly involving the cytoskeleton), and docking and fusion with the plasma membrane (involving components of the SNAP and SNARE family of proteins and associated regulatory proteins) (52). In the basal state, a large proportion of GLUT4 in skeletal muscle from type 2 diabetic subjects sediments in a denser sucrose gradient fraction than usual (18). GLUT4 does not appear to translocate normally from this dense fraction after insulin stimulation. This impairment may be due to anomalous sorting of GLUT4, resulting in its inability to reach its normal reservoir and highly insulin-sensitive intracellular compartment. Alternatively, the dense GLUT4 fraction may actually be associated with the plasma membrane. Thus, an impairment may occur at a late stage in GLUT4 trafficking (i.e., during the fusion of GLUT4 vesicles), which leads to deficient GLUT4 exposure at the cell surface. The difficult issue of resolving the localization of GLUT4 in muscle and in subcellular fractions derived from muscle is compounded by its presence in at least two locations (the sarcolemma and the T-tubules) (53,54). The photolabel we have used to detect GLUT4 will detect this protein at the sarcolemma and T-tubule cell surface membranes (55). In the future, it may be possible to resolve whether GLUT4 translocation at one or both of these locations is insulin resistant.

In conclusion, impaired insulin action on glucose transport in skeletal muscle from type 2 diabetic patients results from reduced cell surface GLUT4 content, as measured by using a newly described photolabel. Nevertheless, the defect in glucose transport was less severe, suggesting another transporter may contribute to insulin-stimulated glucose transport. In response to hypoxia, cell surface GLUT4 content and glucose transport activity are reduced in type 2 diabetic patients. This finding suggests that the glucose transport defects in skeletal muscle from type 2 diabetic patients are not limited to impaired insulin signal transduction. Thus, an impairment at or beyond a point of convergence of the insulin- and noninsulin-mediated signaling pathways or a defect in a common step mediating GLUT4 vesicle traffic appears to play a role in the pathogenesis of insulin resistance in type 2 diabetes. Whether these defects are a primary cause of type 2 diabetes remains to be determined.

Note added in proof. Ibberson et al. (56) have now described the cloning and functional characterization of a novel glucose transporter (GLUTX1) that is expressed in the central nervous system and insulin-sensitive tissue. This or another novel GLUT may account for the discrepancy between reductions in cell surface GLUT4 and transport activity reported here.

ACKNOWLEDGMENTS

This study was supported by a Junior Individual and a project grant from the Foundation for Strategic Research and grants from the Swedish Society for Medical Research, the Novo-Nordisk Foundation, the Swedish Diabetes Association, the Marcus and Amalia Wallenbergs Foundation, the Swedish Medical Research Council (9517, 11945, 11823, and 12211), the Medical Research Council of the United Kingdom, and the Wellcome Trust.

We are indebted to M. Fernström, E.-L. Olausson, and H. Tetzlaf for excellent technical assistance.

REFERENCES

1. DeFronzo RA, Jocot E, Jequier E, Maeder E, Wahren J, Felber JR: The effect of insulin on the disposal of intravenous glucose. *Diabetes* 30:1000–1007, 1981
2. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J: Effects of insulin on peripheral and splanchnic glucose-metabolism in non-insulin-dependent (type-2) diabetes-mellitus. *J Clin Invest* 76:149–155, 1985
3. Andréasson K, Galuska D, Thörne A, Sonnenfeld T, Wallberg-Henriksson H: Decreased insulin-stimulated 3–O-methylglucose transport in in vitro incubated muscle strips from type 2 diabetic subjects. *Acta Physiol Scand* 142:255–260, 1991
4. Zierath JR, Galuska D, Nolte LA, Thörne A, Kristensen JS, Wallberg-Henriksson H: Effects of glycemia on glucose transport in isolated skeletal muscle from patients with NIDDM: in vitro reversal of muscular insulin resistance. *Diabetologia* 37:270–277, 1994
5. Zierath JR: In vitro studies of human skeletal muscle: hormonal and metabolic regulation of glucose transport. *Acta Physiol Scand* 155 (Suppl. 626):1–96, 1995
6. Kelley DE, Mintun MA, Watkins SC, Simoneau J-A, Jadali F, Fredrickson A, Beattie J, Theriault R: The effect of non-insulin-dependent diabetes mellitus and obesity on glucose transport and phosphorylation in skeletal muscle. *J Clin Invest* 97:2705–2713, 1996
7. Bonadonna RC, Del Prato S, Bonora E, Saccomani MP, Gulli G, Natali A, Frascerra S, Pecori N, Ferrannini E, Bier D, Cobelli C, DeFronzo RA: Roles of glucose transport and glucose phosphorylation in muscle insulin resistance of NIDDM. *Diabetes* 45:915–925, 1996
8. Kahn CR: Insulin action, diabetogenes, and the cause of type 2 diabetes. *Diabetes* 43:1066–1084, 1994
9. Nyomba BL, Ossowski VM, Bogardus C, Mott DM: Insulin sensitive tyrosine kinase: relationship with in vivo insulin action in humans. *Am J Physiol* 258:E964–E974, 1990
10. Maegawa H, Shigeta Y, Egawa K, Kobayashi M: Impaired autophosphorylation of insulin receptors from abdominal skeletal muscles in nonobese subjects with NIDDM. *Diabetes* 40:815–819, 1991
11. Nolan JJ, Freidenberg G, Henry R, Reichart D, Olefsky JM: Role of human skeletal muscle insulin receptor kinase in the in vivo insulin resistance of non-insulin-dependent diabetes mellitus and obesity. *J Clin Endocrinol Metab* 78:471–477, 1994
12. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL: Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* 95:2195–2204, 1995
13. Björnholm M, Kawano Y, Lehtihet M, Zierath JR: Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. *Diabetes* 46:524–527, 1997
14. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H: Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes* 47:1281–1286, 1998
15. Gumà A, Zierath JR, Wallberg-Henriksson H, Klip A: Insulin induces translocation of GLUT4 glucose transporters in human skeletal-muscle. *Am J Physiol* 31:E613–E622, 1995
16. Zierath JR, He L, Gumà A, Odegaard-Wahlstrom E, Klip A, Wallberg-Henriksson H: Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* 39:1180–1189, 1996
17. Vogt B, Muhlbacher C, Carrascosa J, Obermaier-Kusser B, Seffer E, Mushack J, Pongratz D, Haring HU: Subcellular-distribution of GLUT4 in the skeletal muscle of lean type 2 (non-insulin-dependent) diabetic patients in the basal state. *Diabetologia* 35:456–463, 1992
18. Garvey WT, Maianu L, Zhu JH, Brechtel-Hook G, Wallace P, Baron AD: Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *J Clin Invest* 101:2377–2386, 1998
19. Lund S, Holman GD, Schmitz O, Pedersen O: Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci U S A* 92:5817–5821, 1995
20. Yeh J-I, Gulve EA, Rameh L, Birnbaum MJ: The effects of wortmannin on rat skeletal muscle: dissociation of signaling pathways for insulin-activated and contraction-activated hexose transport. *J Biol Chem* 270:2107–2111, 1995
21. Lee AD, Hansen PA, Holloszy JO: Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose-transport activity in skeletal-muscle. *FEBS Lett* 361:51–54, 1995
22. Vavvas D, Apazidis A, Saha AK, Gamble J, Patel A, Kemp BE, Witters LA, Ruderman NB: Contraction-induced changes in acetyl-CoA carboxylase and 5′-AMP- activated kinase in skeletal muscle. *J Biol Chem* 272:13255–13261, 1997
23. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ: Evidence for 5′-AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47:1369–1373, 1998
24. Youn JH, Gulve EA, Holloszy JO: Calcium stimulates glucose-transport in skeletal-muscle by a pathway independent of contraction. *Am J Physiol* 260:C555–C561, 1991
25. Youn JH, Gulve EA, Henriksen EJ, Holloszy JO: Interactions between effects of W-7, insulin, and hypoxia on glucose transport in skeletal muscle. *Am J Physiol* 36:R888–R894, 1994
26. Cartee GD, Douen AG, Ramlal T, Klip A, Holloszy JO: Stimulation of glucose-transport in skeletal-muscle by hypoxia. *J Appl Physiol* 70:1593–1600, 1991
27. Zierath JR, Houseknecht KL, Gnudi L, Kahn BB: High fat feeding impairs insulin-stimulated GLUT4 recruitment via an early insulin-signaling defect. *Diabetes* 46:215–223, 1997
28. Zierath JR, Tsao T-S, Stenbit AE, Ryder JW, Galuska D, Charron MJ: Restoration of hypoxia-stimulated glucose uptake in GLUT4-deficient muscles by muscle-specific GLUT4 transgenic complementation. *J Biol Chem* 273:20910–20915, 1998
29. Goodyear LJ, Hirshman MF, Napoli R, Calles J, Markuns JF, Ljungqvist O, Horton ES: Glucose ingestion causes GLUT4 translocation in human skeletal muscle. *Diabetes* 45:1051–1056, 1996
30. Kennedy JW, Hirshman MF, Gervino EV, Ocel JV, Forse RA, Hoenig SJ, Aronson D, Goodyear LJ, Horton ES: Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. *Diabetes* 48:1192–1197, 1999
31. Holman GD, Kozka IJ, Clark AE, Flower CJ, Saltis J, Habberfield AD, Simpson IA, Cushman SW: Cell surface labeling of glucose transporter isoform GLUT4 by bis-bannose photolabel: correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J Biol Chem*

265:18172–18179, 1990
32. Lund S, Holman GD, Schmitz O, Pedersen O: GLUT4 content in the plasma membrane of rat skeletal muscle: comparative-studies of the subcellular fractionation method and the exofacial photolabeling technique using ATB-BMPA. *FEBS Lett* 330:312–318, 1993
33. Lund S, Holman GD, Zierath JR, Rincon J, Nolte LA, Clark AE, Schmitz O, Pedersen O, Wallberg-Henriksson H: Effect of insulin on GLUT4 cell-surface content and turnover rate in human skeletal muscle as measured by the exofacial bis-mannose photolabeling technique. *Diabetes* 46:1965–1969, 1997
34. Koumanov F, Yang J, Jones AE, Hatanaka Y, Holman GD: Cell-surface biotinylation of GLUT4 using bis-mannose photolabels. *Biochem J* 330:1209–1215, 1998
35. DeFronzo RA, Tobin JD, Anders R: Glucose clamp technique: a model for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214–E223, 1979
36. Miles J, Glasscock R, Aikens J, Gerich J, Haymond M: A microfluorometric method for the determination of free fatty acid in plasma. *J Lipid Res* 24:96–99, 1983
37. Wallberg-Henriksson H, Gunnarsson R, Henriksson J, Östman J, Wahren J: Influence of physical training on formation of muscle capillaries in type 1 diabetes. *Diabetes* 33:851–857, 1984
38. Wallberg-Henriksson H, Zetan N, Henriksson J: Reversibility of decreased insulin-stimulated glucose transport capacity in diabetic muscle with in vitro incubation: insulin is not required. *J Biol Chem* 262:7665–7671, 1987
39. Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnieli E, Salans LB, Cushman SW: Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. *Biochim Biophys Acta* 763:393–407, 1983
40. Pedersen O, Bak JF, Andersen PH, Lund S, Moller DE, Flier JS, Kahn BB: Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes* 39:865–870, 1990
41. Jones MN, Nickson JK: Monosaccharide transport proteins of the human erythrocyte membrane. *Biochim Biophys Acta* 650:1–20, 1981
42. Lund S, Flyvbjerg A, Holman GD, Larsen FS, Pedersen O, Schmitz O: Comparative effects of IGF-1 and insulin on the glucose-transporter system in rat muscle. *Am J Physiol* 267:E461–E466, 1994
43. Stenbit AE, Burcelin R, Katz EB, Tsao T-S, Gautier N, Charron MJ, Le Marchand-Brustel Y: Diverse effects of GLUT4 ablation on glucose uptake and glycogen synthesis in red and white skeletal muscle. *J Clin Invest* 98:629–634, 1996
44. Ryder JW, Kawano Y, Chibalin AV, Rincon J, Tsao TS, Stenbit AE, Combatsiaris T, Yang J, Holman GD, Charron MJ, Zierath JR: In vitro analysis of the glucose-transport system in GLUT4 null skeletal muscle. *Biochem J* 342:321–328, 1999
45. Krook A, Björnholm M, Jiang X-J, Galuska D, Fahlman R, Myers M, Wallberg-Henriksson H, Zierath JR: Characterization of insulin signaling and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes* 49:284–292, 2000
46. Jhun BH, Rampal AL, Liu H, Lachaal M, Jung CY: Effects of insulin on steady-state kinetics of GLUT4 subcellular distribution in rat adipocytes: evidence of constitutive GLUT4 recycling. *J Biol Chem* 267:17710–17715, 1992
47. Satoh S, Nishimura H, Clark AE, Kozka IJ, Vannucci SJ, Simpson IA, Quon MJ, Cushman SW, Holman GD: Use of bis-mannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells: evidence that exocytosis is a critical site of hormone action. *J Biol Chem* 268:17820–17829, 1993
48. Tosh D, Clark AE, Pryor PR, Holman GD: Altered GLUT4 subcellular trafficking in primary adipose cell cultures: trafficking kinetic evidence (Abstract). *Mol Biol Cell* 7:2637, 1996
49. Brozinick JT, Birnbaum MJ: Insulin, but not contraction, activates Akt/PKB in isolated rat skeletal muscle. *J Biol Chem* 273:14679–14682, 1998
50. Lund S, Pryor PR, Østergaard S, Schmitz O, Pedersen O, Holman GD: Evidence against protein kinase B as a mediator of contraction-induced glucose transport and GLUT4 translocation in rat skeletal muscle. *FEBS Lett* 425:472–474, 1998
51. Widegren U, Jiang XJ, Krook A, Chibalin AV, Björnholme M, Tally M, Roth RA, Henriksson J, Wallberg-Henriksson H, Zierath JR: Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. *FASEB J* 12:1379–1389, 1998
52. Holman GD, Kasuga M: From receptor to transporter: insulin signaling to glucose transport. *Diabetologia* 40:991–1003, 1997
53. Wang WC, Hansen PA, Marshall BA, Holloszy JO, Mueckler M: Insulin unmasks a COOH-terminal GLUT4 epitope and increases glucose transport across T-tubules in skeletal muscle. *J Cell Biol* 135:415–430, 1996
54. Ploug T, van Deurs B, Ai H, Cushman SW, Ralston E: Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. *J Cell Biol* 142:1429–1446, 1998
55. Dudek RW, Dohm GL, Holman GD, Cushman SW, Wilson CM: Glucose-transporter localization in rat skeletal muscle: autoradiographic study using ATB-[3-H]-BMPA photolabel. *FEBS Lett* 339:205–208, 1994
56. Ibberson M, Uldry M, Thorens B: GLUTX1, a novel mammalian glucose transporter expressed in the central nervous system and insulin sensitive tissues. *J Biol Chem* 275:4607–4612, 2000