Glycemic Improvement in Diabetic db/db Mice by Overexpression of the Human Insulin-regulatable Glucose Transporter (GLUT4)


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Abstract

The effects of increased GLUT4 (insulin-regulatable muscle/fat glucose transporter) expression on glucose homeostasis in a genetic model of non-insulin-dependent diabetes mellitus were determined by expressing a human GLUT4 transgene (hGLUT4) in diabetic C57BL/KsJ-db/db mice. A genomic hGLUT4 construct was microinjected directly into pronuclear murine embryos of db/+ matings to maintain the inbred background. Four lines of hGLUT4 transgenic mice were bred to homozygosity at the db locus and all showed a marked reduction of both fasting and fed plasma glucose levels (to ~50 and 360 mg/dl, respectively) compared with age-matched nontransgenic db/db mice (~215 and 550 mg/dl, respectively), as well as an enhanced disposal of an oral glucose challenge. In situ immunocytochemical localization of GLUT4 protein in muscle from hGLUT4 db/db mice showed elevated plasma membrane- and intracellular GLUT4 protein in the basal state, which markedly increased after an insulin/glucose injection. In contrast, nontransgenic db/db mice had low levels of plasma membrane- and intracellular GLUT4 protein in the basal state with a relatively small increase after an insulin/glucose challenge. Since the intracellular GLUT4 levels in db/db mice were similar to nontransgenic db/+ mice, the glucose transport defect in db/db mice is at the level of glucose transporter translocation. Together, these data demonstrate that GLUT4 upregulation overcomes the glucose transporter translocation defect and alleviates insulin resistance in genetically diabetic mice, thus resulting in markedly improved glycemic control. (J. Clin. Invest. 1995; 95:1512-1518.) Key words: non-insulin-dependent diabetes mellitus • genetic models • basal metabolism • gene expression • upregulation

Introduction

Non-insulin-dependent diabetes mellitus (NIDDM)1 is a metabolic disease that affects ~5% of the population of the Western world. NIDDM is characterized by hyperglycemia in both the fed and fasted states and is predominantly associated with obesity. The disease is manifested by defects in both insulin secretion from the pancreas and insulin action in peripheral target tissues (i.e., liver, skeletal muscle, and adipose tissue). Skeletal muscle is the major site of post-prandial peripheral glucose disposal, and muscle from NIDDM patients displays markedly impaired glucose uptake in response to insulin, although the precise nature of the defect is unknown (1-3). The defect in glucose transport cannot be explained by a simple reduction in the level of gene expression of the major muscle glucose transporter isoform (GLUT4), as little or no reduction in skeletal muscle GLUT4 content has been found in NIDDM patients (3-6). Several lines of evidence suggest that elevated glucose levels directly affect insulin sensitivity in target tissues and contribute to the dire long-term diabetic complications such as cardiovascular disease, retinopathy, neuropathy, and nephropathy (7, 8).

db/db mice are a genetic model of NIDDM that display many of the characteristics of the human disease including hyperglycemia, insulin resistance, and obesity (9-11). Importantly, as in human NIDDM, db/db mice have a marked decrease in skeletal muscle glucose utilization due to a major defect in glucose transport that is not accompanied by significant alterations in GLUT4 expression (12, 13). In this study, the possible beneficial effects of GLUT4 overexpression in this murine NIDDM model were tested by producing transgenic mice that express a human GLUT4 transgene (14). These data indicate that GLUT4 upregulation in this genetic model of NIDDM reduced hyperglycemia and resulted in improved glycemic control.

Methods

Transgenic mice. Pronuclear transgenic mice were produced by established protocols (15, 16), however, embryos were obtained from the inbred, mutant mouse strain, C57BL/KsJ-m+/m-db (Jackson Laboratories, Bar Harbor, ME), in which the recessive diabetic mutation, db, is linked in replication to the recessive coat color mutation, misty, m (17). The linking of db and m mutations in repulsion allows for the identification of offspring genotypically wild-type (+/+) at the db locus by the presence of the recessive misty (m/m) coat color (17). An

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*Abbreviations used in this paper: db, diabetes; GLUT4, insulin-regulatable muscle/fat glucose transporter; hGLUT4, human GLUT4; m, misty; NIDDM, non-insulin-dependent diabetes mellitus; nt, nucleotide.
11.8-kb (Not fragment) human genomic GLUT4 transgene including 5.3 kb of 5′ flanking sequence and an SV40 polyadenylation sequence (14) was microinjected at ~2.0 μg/ml into pronuclei of embryos derived from matings of C57BL/KsJ-m+/+db males and superovulated females. 400 injected embryos were transferred to pseudopregnant CD-1 females. Of 135 offspring, 13 founder transgenic mice were identified by polymerase chain reaction (PCR) of total genomic DNA (data not shown). We observed two founders with the recessive, obese phenotype (+/db/+db) and two founders with the recessive misty coat color (m+/m+). The remaining nine founders were m+/+db with black coat and lean phenotype. Independent lines were established by backcrossing lean founder mice with C57BL/KsJ-m+/+db mates.

**R**Nase protection assay. Total RNA was isolated from heart tissue using RNAsol™B (BIOTECH, Inc, Houston, TX) following the manufacturer's directions. The human-specific GLUT4 probe, P JW3 (14), was linearized with EcoR1 and its T3 promoter was used to generate a 32P-labeled antisense probe. The RNA from each sample was hybridized for 16 h at 55°C, treated with RNase A, and electrophoresed on a 6% polyacrylamide/7.5 M urea gel.

**Immunoblotting.** Postnuclear membrane fractions were prepared from frozen hearts and adipose tissue as described previously (18). Samples were solubilized in Laemmli loading buffer, and proteins (50 μg protein) were separated on 10% reducing SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were incubated with affinity-purified rabbit anti-GLUT4 polyclonal antibodies (19) overnight at 4°C. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL) according to the manufacturer’s instructions. Bands were quantitated using a MasterScan interpretive densitometer (Scanalytics, a Division of CSPI, San Diego, CA).

**Oral glucose tolerance test and metabolite measurements.** Nonanesthetized GLUT4 transgenic (db/+ and db/db) and age-matched nontransgenic mice were fasted overnight and bled via the orbital sinus (0.025 ml) immediately before administration of an oral glucose load (1 g glucose per kg body wt) by gavage using a syringe equipped with a murine oral feeding needle (20 gauge; Popper & Sons, Inc., New Hyde Park, NY). Mice were subsequently bled after 30, 75, and 120 min. Plasma glucose levels were measured using the VP Super System Autoanalyzer (Abbott Laboratories, North Chicago, IL). Plasma insulin was determined using a radioimmunoassay (Binxas, Portland, ME) with porcine insulin as standard.

**Immunofluorescence.** Nontransgenic and GLUT4 transgenic db/db mice were either fasted overnight and left untreated or allowed to eat ad libitum and then given an intraperitoneal injection of glucose (1 g/kg) and insulin (8 U/kg) as previously described (20, 21). 30 min after the glucose/insulin challenge, mice were anesthetized and immediately perfused through the left ventricle with 25 ml of 4% formaldehyde plus 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 23°C. After fixation in situ, cardiac and quadriceps muscle and pancreas were removed, fixed by immersion for 1 h, and processed for frozen sectioning as previously described (22). Briefly, 0.5-μm frozen sections were incubated at 23°C with 4.8 μg/ml rabbit anti-human GLUT4 IgG (19) for 1 h followed by incubation with 5 μg/ml donkey anti-rabbit IgG conjugated to Texas red (Jackson Laboratories). Optimal exposure time was determined for the insulin/glucose-treated transgenic mice, and all other conditions were photographed with an identical exposure time on a Nikon FXA microscope.

**Results and Discussion**

The relative expression of human GLUT4 (hGLUT4) in eight lines of F1 transgenic mice was determined by RNase protection of total RNA from cardiac tissue (Fig. 1 A). Tissue-specific expression of hGLUT4 RNA was examined by reverse transcriptase-PCR of purified total RNA from four of the lines expressing hGLUT4 levels. In addition to cardiac muscle, appropriate expression (23, 24) of the hGLUT4 transgene was detected in hindquarter skeletal muscle, perirenal brown adipose tissue, and white adipose tissue (epidydimal and subcutaneous for male and female mice, respectively; data not shown). We also detected the hGLUT4 transgene RNA in total brain homogenates from the four lines analyzed, which is consistent with the recent observation that GLUT4 is also expressed at low levels in the brain (25, 26). As expected, the hGLUT4 transgene RNA was not detected in liver, kidney, spleen, or pancreas from hGLUT4 lines 5, 8, or 12, however, the highest expressing line (hGLUT4 line 6; Fig. 1 A) showed inappropriate expression in the kidney and liver, presumably due to chromosomal position effects of the transgene (27). With the exception of hGLUT4 transgene expression in the brain, the present results are consistent with earlier observations in hGLUT4 transgenic mice produced on a nondiabetic hybrid background (14) and confirm that the hGLUT4 transgene is expressed in parallel to the endogenous murine GLUT4 gene. Densitometric analysis of immunoblots indicated that GLUT4 protein was overexpressed four- to sixfold in cardiac muscle and approximately threefold in white adipose tissue in hGLUT4db/db mice compared with nontransgenic db/db mice (Fig. 1, B and C).

The effect of GLUT4 overexpression on body weight of db/+ and db/db mice is shown in Fig. 2. Body weights of hGLUT4 transgenic db/+ mice of both sexes were similar to or slightly less than for nontransgenic db/+ mice up to 40 wk of age, which is consistent with our earlier observations in hGLUT4 transgenic mice produced on a hybrid background (21). Body weights of both female (Fig. 2 A) and male (Fig. 2 B) hGLUT4 transgenic db/db mice were comparable with nontransgenic db/db mice up to ~10 wk of age. However, the nontransgenic db/db mice stopped gaining weight at this age, and in fact slowly lost weight over the remaining course of their lives, consistent with progression of the diabetic phenotype (10). In marked contrast, the hGLUT4db/db transgenic mice continued to gain weight until ~15 wk of age and then maintained this weight until at least 35 wk of age. Since overexpression of GLUT4 in muscle and fat is not a direct cause of obesity as evidenced by the results in hGLUT4db/+ transgenic mice (Fig. 2) and hGLUT4 transgenic hybrid mice (14, 21), it is likely that GLUT4 overexpression in severely insulin-resistant db/db mutant mice at least partially overcomes the insulin resistance and thus enhances the animals' ability to thrive.

The improved weight gain of the hGLUT4db/db transgenic mice was in direct contrast with a previous study in which GLUT4 was selectively overexpressed in adipose tissue using the aP2 promoter in a different genetic mouse background (28). Although there was a marked increase in adiposity in these animals, this did not occur when GLUT4 was overexpressed in both muscle and adipose tissue using the GLUT4 promoter (21). Thus, we hypothesize that either the overexpression of GLUT4 in both muscle and adipose tissue prevents fat cell hyperplasia or that these differences in adiposity arise due to the use of an early developmental aP2 promoter (29) versus the late developmental GLUT4 promoter (30).

In any case, we next determined the functional expression of the hGLUT4 transgene on glucose homeostasis in vivo by assessing the physiological responses of both nondiabetic (db/+ +) and diabetic (db/db) mice to an oral glucose challenge (Fig. 3). 30 min after an oral glucose load (1 g/kg), a substantial increase in plasma glucose levels (approximately twofold) occurred in nontransgenic, nondiabetic db/+ + mice (Fig. 3 A). This increase in circulating glucose levels was transient and gradually declined toward basal levels by 120 min. In contrast, hGLUT4 transgenic nondiabetic db/+ + mice from three overex-
pressing lines displayed reduced fasting plasma glucose, which remained below 100 mg/dl after the glucose load. One hGLUT4 transgenic line did not have reduced fasting plasma glucose compared with the nontransgenic mice, but showed essentially no increase in plasma glucose after the glucose load (hGLUT4 line 8; Fig. 3 A). The male mice used in the experiment shown in Fig. 3 A ranged from 7 to 16 wk of age; similar results were obtained in female mice and also in one line of hGLUT4 db/db male mice (line 8) that was tested at 6 and 90 wk of age (data not shown). Further, as was observed in our previous studies (21, 31), functional expression of the GLUT4 transgene in skeletal and cardiac muscle was confirmed by a two- to fourfold increase in glycogen content in hGLUT4 transgenic mice (Gibbs, E. M., S. C. McCoid, W. J. Zavadoski, J. L. Stock, J. D. McNeish, and R. W. Stevenson, unpublished observations). Thus, high level expression of hGLUT4 protein in non diabetic, db/+ heterozygotes produced a phenotype similar to GLUT4 transgenic mice on a nondiabetic hybrid background (21, 31), and the effect of the transgene was maintained over the course of the animals’ lives.

Nontransgenic db/db mice (8–9 wk of age) had fasting hyperglycemia (~250 mg/dl) that markedly increased up to ~600–700 mg/dl, after the glucose challenge (Fig. 3 B). Remarkably, age-matched db/db mice expressing the hGLUT4
transgene (line 12) had fasting plasma glucose levels of ~50 mg/dl, which typically remained below 100 mg/dl even after the oral glucose challenge (Fig. 3B; similar results were obtained for three other hGLUT4 lines, not shown).

As expected, fed plasma insulin levels were reduced in female transgenic db/+ mice compared with female nontransgenic db/+ mice (28.3±2.5 vs 39.5±3.4 μU/ml; \( P < 0.025 \); mice were 6–11 wk of age) and in male transgenic mice db/+ compared with nontransgenic db/+ mice (37.1±3.1 vs 65.5±9.3 μU/ml; \( P < 0.005 \); mice were 6–10 wk of age). The insulin-lowering effect in the transgenic mice presumably occurs in response to the hypoglycemia produced by increased...

**Figure 2.** Body weight versus age in nontransgenic and transgenic hGLUT4db/+ and db/db mice. Body weights of female (A) and male (B) db/+ (squares) and db/db (circles) nontransgenic (open symbols) and hGLUT4 transgenic (closed symbols) mice are presented as means±SEM (\( n = 3–15, \) with a median \( n = 5 \)). Most data points represent means obtained from single measurements of individual mice. However, because of the limiting number of hGLUT4db/db transgenic mice that was available, a subgroup of this phenotype was set aside for this study (\( n = 22 \) and 17, for female and male mice, respectively) and was reweighed at 2-wk intervals covering the 15–35 wk age range.

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glucose disposal. Surprisingly, fed plasma insulin levels in hGLUT4db/db transgenic male and female mice were significantly higher than nontransgenic db/db mice, whereas fasting plasma insulin levels were lower in transgenic db/db mice of both sexes (Table I). The increased fed plasma insulin levels in the transgenic db/db mice likely result from a (partial) protection from the glucose-dependent pancreatic atrophy that occurs in nontransgenic db/db mice as they age (9–11, 32). Along this line, we have observed that the islet surface area in paraffin sections of pancreatic tissue is approximately threefold greater in hGLUT4db/db mice compared with age-matched (~30 wk) nontransgenic db/db mice (Milici, A. J., D. N. Scampoli, S. C. McCoid, J. L. Stock, J. D. McNeill, R. W. Stevenson, and E. M. Gibbs, unpublished observations). Since inappropriate GLUT4 expression in β cells might be expected to alter insulin secretion, it was important to demonstrate that this did not occur. hGLUT4 RNA was not detected in pancreatic RNA using the sensitive reverse transcriptase-PCR methodology. In addition, no difference in GLUT4 expression was observed in frozen sections of pancreatic tissue from transgenic mice compared with nontransgenic mice as assessed by immunofluorescence (data not shown). Taken together, these data suggest that expression of the hGLUT4 transgene in an appropriate tissue-specific manner in db/db mice results in more functional pancreatic β cells that can efficiently respond to the partially elevated fed plasma glucose levels than nontransgenic db/db mice. These data suggest that lowering plasma glucose by increased GLUT4 expression reduces stress on the pancreas and maintains its functional integrity, thus preventing the mice from progressing into the late hypoinsulinemic stage of NIDDM.

To assess GLUT4 subcellular localization in vivo under fasting and maximally insulin-stimulated states (20, 21), we examined the cardiac myocyte distribution of the GLUT4 protein by immunofluorescence using a GLUT4 specific antibody (19) coupled with a Texas red–conjugated secondary antibody.
Figure 4. Immunofluorescent localization of cardiac myocyte GLUT4 protein expression in nontransgenic and transgenic hGLUT4 db/db mice. Female nontransgenic (A and C) and hGLUT4 line 8 transgenic (B and D) db/db mice (9 wk of age) were either fasted overnight and left untreated (A and B) or allowed to feed ad libitum and given an intraperitoneal injection of insulin (8 U/kg) and glucose (1 g/kg) (C and D). Arrowheads mark several locations of punctate cytoplasmic staining (A and B) in myocytes from the fasted animals, and arrows mark isolated myocytes in which the entire membrane was labeled in a fasted transgenic animal (B). Arrows mark locations of membrane-associated GLUT4 (C and D) in myocytes after insulin/glucose treatment. Insulin induced a very patchy translocation of GLUT4 in the nontransgenic mice (C), whereas in the transgenic mice (D) there was an intense uniform labeling of the entire myocyte plasmalemma. Essentially identical results were also obtained from db/db hGLUT4 line 12 transgenic mice (8 wk of age; data not shown). No significant fluorescent signal could be detected in any tissue sections from either nontransgenic or transgenic mice labeled with equal concentrations of nonimmune rabbit IgG (data not shown). ×900.

Cardiac muscle tissue from fasted nontransgenic db/db mice exhibited a punctate cytoplasmic GLUT4 immunofluorescence (Fig. 4 A). Both the level of GLUT4 immunofluorescence and localization in the basal state were similar to our previous observations in normal mice (21). With insulin and glucose treatment, nontransgenic db/db mice showed a minimal redistribution of GLUT4 immunofluorescence from the condensed cytoplasmic vesicles to the plasmalemma (Fig. 4 C). This contrasts with our previous data indicating substantial GLUT4 translocation in nondiabetic mice (21) and is a direct demonstration that one manifestation of the insulin resistance in the db/db mice is a GLUT4 translocation defect. Overexpression of GLUT4 protein in vivo resulted in a specific immunofluorescent signal that was readily detected in both the plasma membrane and intracellular vesicles from cardiac myocytes of the fasted non–insulin-treated hGLUT4 db/db line 8 transgenic mice (Fig. 4 B). Furthermore, GLUT4 db/db transgenic mice treated with insulin and glucose demonstrated a marked increase in cell surface GLUT4 protein content with an accompanying decrease in the punctate intracellular GLUT4 immunofluorescence (Fig. 4 D). Qualitatively and quantitatively similar immunofluorescent data were obtained in quadriceps muscles obtained from the same mice used in these experiments (data not shown), which indicates that the cardiac myocyte analysis is representative of GLUT4 translocation in skeletal muscle.

Quantitative data obtained by image analysis of cardiac myocytes from nontransgenic db/db mice demonstrated only a 1.2-fold increase in plasmalemma GLUT4 immunofluorescence after insulin stimulation. In contrast, basal levels of plasmalemma GLUT4 in the transgenic db/db mice were much greater than those observed in insulin-treated nontransgenic mice and increased more than threefold after the insulin chal-
lenge. These data indicate that in the hGLUT4/db/db transgenic mice relatively high levels of GLUT4 protein reside at the plasma membrane in the basal state and that insulin directly induces an increase in plasma membrane–associated human GLUT4 protein in a manner consistent with translocation from an intracellular vesicular pool. This GLUT4 redistribution occurs in spite of the insulin resistance (9–13) and translocation defect (see above) in the nontransgenic db/db mice.

Thus, if the defect in insulin action in db/db mice occurs in the insulin signaling pathway, it does not appear to be rate limiting in preventing GLUT4 translocation from the intracellular pool to the plasma membrane. Since the defect in the insulin signaling pathway in db/db mice appears to be overcome by increased GLUT4 expression, it is possible that increased glucose disposal reduces hyperglycemia and the "glucose toxicity" proposed by DeFronzo (7) as a contributing factor to insulin resistance.

In summary, these data demonstrate that high level expression of the human insulin responsive GLUT4/muscle-fat specific facilitative glucose transporter transgene in a genetic model of NIDDM results in a high level of cell surface GLUT4 protein localization. Additionally, the increased GLUT4 expression resulted in a restoration of insulin-stimulated GLUT4 translocation from the intracellular storage site to the plasma membrane. As a consequence, after a large oral glucose load, circulating glucose levels were markedly reduced in the hGLUT4 transgenic db/db mice compared with the nontransgenic db/db mice, indicating a substantially greater degree of glycemic control. Most importantly, these results suggest that increasing GLUT4 levels at the plasma membrane by either genetic manipulation or by pharmacologic intervention may be an effective therapy for human NIDDM as improved glycemic control occurs even in the presence of severe insulin resistance and pancreatic defects.

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References