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 fasted 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-associated 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–associated 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, 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+/+ db (Jackson Laboratories, Bar Harbor, ME), in which the recessive diabetic mutation, db, is linked in repulsion 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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1. 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.
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 high 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 (epididymal 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 nonobese diabetic 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 hGLUT4/db/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 hGLUT4/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 hGLUT4/db/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 hGLUT4/db/+ 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 hGLUT4/db/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. 3A). The male mice used in the experiment shown in Fig. 3A ranged from 7 to 16 wk of age; similar results were obtained in female mice and also in one line of hGLUT4 db/db 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 nondo-abetic, db/+ heterozygotes produced a phenotype similar to GLUT4 transgenic mice on a nondoabetic 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. 3B). Remarkably, age-matched db/db mice expressing the hGLUT4

Figure 1. Expression of hGLUT4 in transgenic db/db mice. (A) RNase protection assay of hGLUT4 lines. 15 µg of heart total RNA was probed with 5 × 10⁵ cpm of the human GLUT4 specific probe, pJW3, to determine hGLUT4 mRNA expression as described in Methods. Lanes 1, 2, 3, 4, 5, 7, 8, and 9 correspond to individual mice (7–15 wk of age) from hGLUT4 lines 4, 3, 7, 5, 6, 12, 8, and 11, respectively. Lane 6 is a nontransgenic negative control mouse, while lane 10 is a positive control mouse from the previously characterized hGLUT4C line (14, 21, 31). The transgenic GLUT4 protected fragment is 241 nucleotides (nt) and migrates just ahead of a 250-nt size marker (lane 11). The dried gel was exposed to x-ray film at −70°C with an intensifying screen for 3 d. (B) Immunoblot analysis of hGLUT4 transgenic line 8. 50 µg of adipose tissue (top) or cardiac muscle (bottom) postnuclear membrane protein was analyzed for GLUT4 protein content using anti-GLUT4 antibodies as described in Methods. NTG indicates three samples from female db/db nontransgenic mice, and LINE8 indicates three samples from female hGLUT4 line 8 db/db transgenic mice. (C) Relative expression of GLUT4 protein. GLUT4 bands from the immunoblots of female mice shown in B and also from immunoblots of cardiac muscle of male nontransgenic (n = 3) and hGLUT4db/db transgenic (n = 5) mice were quantitated by densitometric analysis. Lightly shaded bars represent nontransgenic mice, and hatched bars represent transgenic mice. All mice analyzed were between 9 and 12 wk of age. Significant differences from sex-matched nontransgenic mice are indicated, * P < 0.05, ** P < 0.01, and *** P = 0.07.
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 hGLUT4 db/+ 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 hGLUT4 db/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.

Glycemic Improvement in db/db Transgenic Mice 1515
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 1). 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. McNeish, 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.

Table 1. Comparison of Plasma Glucose and Insulin Levels between Nontransgenic db/db and hGLUT4 Transgenic db/db Mice

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<th>Male</th>
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<td>Fed glucose (mg/dl)</td>
<td>Fasting glucose (mg/dl)</td>
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<td>db/db</td>
<td>hGLUT4</td>
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<td>n</td>
<td>22</td>
<td>14</td>
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<tr>
<td>537±18</td>
<td>363±44*</td>
<td>566±30</td>
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<td>110±7</td>
<td>69±9*</td>
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Data from transgenic mice between 7 and 11 wk of age from hGLUT4 lines 5, 6, 8, and 12 were pooled for these analyses. The value in parentheses indicates the number of animals used for each analysis. Significant differences from age- and sex-matched nontransgenic mice are indicated, *P < 0.005, **P < 0.0005.
Cardiac muscle tissue from fasted nontransgenic db/db mice exhibited a punctate cytoplasmic GLUT4 immunofluorescence (Fig. 4A). 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. 4C). 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. 4B). 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. 4D). 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