Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle

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Carvalho, Eugenia, Ko Kotani, Odile D. Peroni, and Barbara B. Kahn. Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle. Am J Physiol Endocrinol Metab 289: E551–E561, 2005. First published May 31, 2005; doi:10.1152/ajpendo.00116.2005.—Adipose tissue plays an important role in glucose homeostasis and affects insulin sensitivity in other tissues. In obesity and type 2 diabetes, glucose transporter 4 (GLUT4) is downregulated in adipose tissue, and glucose transport is also impaired in muscle. To determine whether overexpression of GLUT4 selectively in adipose tissue could prevent insulin resistance when glucose transport is impaired in muscle, we bred muscle GLUT4 knockout (MG4KO) mice to mice overexpressing GLUT4 in adipose tissue (AG4Tg). Overexpression of GLUT4 in fat not only normalized the fasting hyperglycemia and glucose intolerance in MG4KO mice, but it reduced these parameters to below normal levels. Glucose infusion rate during a euglycemic clamp study was reduced 46% in MG4KO compared with controls and was restored to control levels in AG4Tg-MG4KO. Similarly, insulin action to suppress hepatic glucose production was impaired in MG4KO mice and was restored to control levels in AG4Tg-MG4KO. 2-Deoxyglucose uptake during the clamp was increased approximately twofold in white adipose tissue but remained reduced in skeletal muscle of AG4Tg-MG4KO mice. AG4Tg and AG4Tg-MG4KO mice have a slight increase in fat mass, a twofold elevation in serum free fatty acids, an ~50% increase in serum leptin, and a 50% decrease in serum adiponectin. In MG4KO mice, serum resistin is increased 34% and GLUT4 overexpression in fat reverses this. Overexpression of GLUT4 in fat also reverses the enhanced clearance of an oral lipid load in MG4KO mice. Thus overexpression of GLUT4 in fat reverses whole body insulin resistance in MG4KO mice without restoring glucose transport in muscle. This effect occurs even though AG4Tg-MG4KO mice have increased fat mass and low adiponectin and is associated with normalization of elevated resistin levels.

Recent studies have challenged long-held concepts about the pathogenesis of type 2 diabetes. It is still clear that insulin resistance is a major factor in the pathogenesis of type 2 diabetes (35), but the role of individual insulin target tissues (skeletal muscle, liver, and adipose tissue) has come into question (22, 26). In particular, studies in which insulin receptor signaling has been altered in specific insulin target tissues suggest that insulin action in the liver may play a dominant role in glucose homeostasis (18). Interestingly, insulin receptors in muscle appear to play a more minor role in glucose homeostasis but affect adiposity (6, 16, 18, 22). However, the relatively subtle effect of genetic alterations in insulin receptors in muscle on glucose homeostasis may be partially because of compensation by insulin-like growth factor (IGF) signaling in the muscle (18, 30). For example, expression of dominant-negative IGF-I receptors in muscle, which impairs both IGF-I and insulin signaling, results in diabetes (7, 14). Furthermore, the absence of glucose transporter (GLUT) 4 in muscle causes mild diabetes, indicating that insulin action on glucose transport in muscle is important for glucose homeostasis (39). Various lines of evidence indicate that insulin action and glucose transport in adipose tissue are important determinants of systemic insulin sensitivity and glucose homeostasis (3, 5, 26, 32).

The rate-limiting step for glucose utilization in muscle and adipose tissue is the transmembrane transport of glucose mediated by GLUT4 (11), which is expressed primarily in skeletal muscle, heart, and adipocytes. GLUT4 gene expression is downregulated in adipocytes but not in muscle from humans with type 2 diabetes and/or obesity (8, 9, 38). This adipose-specific downregulation of GLUT4 was not thought to play a major role in glucose homeostasis several years ago, because of the relatively small amount of glucose taken up by adipose tissue.

To understand the importance of insulin action on glucose transport in muscle and adipose tissue, we used Cre-LoxP gene targeting to selectively target GLUT4 expression in adipocytes or muscle. Adipose-specific GLUT4 knockout mice develop insulin resistance and have an increased risk of type 2 diabetes (3). Whereas the genetic defect is limited to adipose tissue, they become insulin resistant secondarily in muscle and liver. Adipose tissue can indirectly influence insulin action in other tissues through multiple mechanisms, including release of fatty acids, cytokines, and hormones (12).

In adipose-GLUT4 knockout mice, the impaired insulin action in muscle and liver is most likely because of alteration in adipocyte-secreted molecules, since insulin action in muscle from these mice is normal ex vivo (3). Conversely, mice that overexpress GLUT4 selectively in adipose tissue have enhanced glucose tolerance, insulin sensitivity, fasting hypoglycemia, and hypoinsulinemia, even though they have mild obesity (32). Therefore, increasing GLUT4 expression in adipose tissue without any other direct manipulation of the pathways for insulin signaling or cellular metabolism enhances whole body glucose utilization.

Mice lacking GLUT4 selectively in muscle have insulin resistance and diabetes from a young age. Although initially the insulin resistance is only in muscle, subsequently they become insulin resistant also in adipose tissue and liver (17, 31).
We hypothesized that overexpression of GLUT4 in adipose tissue might compensate for the insulin resistance caused by reduction of GLUT4 expression in skeletal muscle and reverse the fasting hyperglycemia and mild diabetes in these mice.

In the present study, we crossed muscle-specific GLUT4 knockout (MG4KO) mice with mice overexpressing GLUT4 specifically in adipose tissue (AG4Tg) and found that overexpression of GLUT4 in adipose tissue rescues the glucose intolerance and insulin resistance in mice lacking GLUT4 in muscle. This is in spite of the fact that these mice have mild obesity, elevated serum free fatty acids (FFA), and reduced serum adiponectin. This could have important implications for the insulin resistance of obesity and type 2 diabetes mellitus since GLUT4 is downregulated in adipocytes and GLUT4 subcellular localization and/or function is impaired in muscle in these conditions, leading to impaired insulin-stimulated glucose transport in both tissues (10).

METHODS

Animals. Transgenic mice with expression of Cre recombinase driven by the muscle creatine kinase (MCK) promotor (6) were crossed with mice carrying a GLUT4 allele with exon 10 flanked by LoxP sites (GLUT4 Lox mice) that were created by homologous recombination (2). Muscle-specific GLUT4 knockout mice were generated by this cross and were identified by PCR analysis of genomic DNA (39). The genetic background of these mice was a mixture of C57Bl6, 129, and FVB strains. Transgenic mice overexpressing GLUT4 selectively in adipose tissue were made using the adipose-specific promoter/enhancer from the fatty acid-binding protein gene, ap2 (32). These FVB animals were all heterozygous for the transgene. Mice that were heterozygous for GLUT4 in muscle (MCK-Cre<sup>+/–</sup>-GLUT4 Lox<sup>+/–</sup>) were crossed to the adipose-GLUT4 overexressor, carrying one GLUT4 Lox allele (AG4Tg/GLUT4 Lox<sup>+/–</sup>), giving rise to six different genotypes (see results). These transgenic animals have an outbred background of C57BL/6, 129, and FVB. The mice were housed in a pathogen-free environment on a 14:10-h light-dark cycle and fed standard rodent chow. The Institutional Animal Care and Use Committee (Beth Israel Deaconess Medical Center, Boston, MA) approved all the studies.

Glucose transport in isolated adipocytes and skeletal muscle. Basal and insulin-stimulated [U-14C]glucose transport in isolated adipocytes was carried out previously (32), under conditions in which glucose uptake reflects transport. Insulin-stimulated glucose transport in extensor digitorum longus (EDL) and soleus muscles was performed with or without 33 nM insulin, in 2-mo-old male mice, after an overnight fast (39).

Metabolite assays. Plasma insulin was measured with the Rat Insulin ELISA Kit, using mouse standards. Serum FFA were measured in fasted animals using the NEFA-C kit (Wako) with oleic acid as the standard. Serum triglyceride levels were measured by the colorimetric enzyme assay (Sigma-Aldrich), lepin was measured with an ELISA Kit (CrystalChem), adiponectin/ACRP30 was measured using an RIA Kit, and resistin was measured using an ELISA kit both from LincoResearch.

Body composition. Female mice were anesthetized (100 mg/kg ketamine-10 mg/kg xylazine) and scanned using dual-energy X-ray absorptiometry (DEXA) to analyze body fat content using a Lunar PIXImus densitometer (LUNAR). Fat mass analyzed by DEXA is overestimated by 20% compared with carcass analysis (25).

Glucose and insulin tolerance tests. Glucose tolerance tests were performed by intraperitoneal injection of glucose (2 g·kg<sup>−1</sup>·body wt<sup>−1</sup>) after an overnight fast (13–15 h). Blood was sampled from the tail vein before injection (time 0) and 15, 30, 60, and 120 min after glucose injection. Insulin tolerance was tested in mice after removal of food for 3–4 h, by intraperitoneal injection of insulin (0.75 U insulin/kg body wt; Eli Lilly). Blood was sampled at 0 (before injection), 15, 30, 45, 60, and 90 min after injection. Blood glucose was measured with a One Touch II glucose meter (Lifescan). For some studies, plasma glucose was measured with the One Touch Ultra glucose meter (Lifescan).

Enzyme activities. AMPK and ACC activities were measured as previously described (23). In brief, to measure isoform-specific AMPK activity in skeletal muscle and liver, we immunoprecipitated AMPK from these tissue lysates (100 μg protein) with specific antibodies against the α<sub>1</sub>- or α<sub>2</sub>-catalytic subunits bound to protein G-Sepharose beads. We measured kinase activity using synthetic “SAMS” peptide and [γ-<sup>32</sup>P]ATP. The activity of ACC in muscle and liver lysates was measured by 14CO2 fixation to acid-stable products in the presence or absence of citrate (2 mM), an allosteric activator of ACC (23).

Hyperinsulinemic-euglycemic clamp. Clamp studies were performed on 3-mo-old male mice and 10-mo-old female mice. Before the study (5 days), mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body wt; Sigma-Aldrich) and xylazine (10 mg/kg body wt; Sigma-Aldrich), and an indwelling catheter (BD) was inserted in the right jugular vein (17). The mice recovered fully from surgery before the in vivo experiments were performed. For the GLUT4-overexpressing mice, hyperinsulinemic-euglycemic clamp experiments were started at 11:00 A.M. after removal of food at 8:00 A.M. For all other mice, food was removed at 8:00 A.M., and experiments were started at 1:00 P.M. To estimate the rate of basal glucose turnover, [3-<sup>3</sup>H]glucose (0.05 μCi/min) was infused for 2 h before the start of the clamp, and a blood sample (20 μl) was taken at the end of this period. After the basal period, the hyperinsulinemic-euglycemic clamp was performed over 120 min. Human insulin (Eli Lilly) was continuously infused at a rate of 15 pmol·kg<sup>−1</sup>·min<sup>−1</sup> (2.5 μU·kg<sup>−1</sup>·min<sup>−1</sup>) to raise plasma insulin within the physiological range. Plasma glucose concentration was measured every 10 min during the clamp, and 40% glucose was infused at variable rates to maintain plasma glucose levels at 7 mM. Insulin-stimulated whole body glucose flux was measured using a continuous infusion of [3-<sup>3</sup>H]glucose (10 μCi bolus, 0.1 μCi/min; NEN Life Sciences) throughout the clamp. Insulin-stimulated glucose uptake in individual tissues in vivo was assessed using 2-deoxy-D-[1-<sup>14</sup>C]glucose (2-14CJDG), as previously described (17). Blood samples (20 μl) were collected before the start and at the end of the clamp to measure plasma insulin concentrations. All infusions were done using microdialysis pumps (CMA/ Microdialysis). At the end of the clamp, tissues were collected and frozen immediately using liquid N<sub>2</sub>.
and stored at −70°C for later analysis. Rates of basal glucose turnover and whole body glucose uptake, hepatic glucose production during the hyperinsulinemic-euglycemic clamp, and glucose uptake in individual tissues were determined as described previously (17).

**Lipid loading test.** A gavage with olive oil was performed as described previously (1). After 4 h of removal of food, mice were given ad libitum access to water, olive oil (6 μl/g body wt) was introduced intragastrically using a gavage needle, and 50 μl blood were collected at 0, 1, 2, 3, and 5 h for determination of triglyceride and FFA concentrations.

**Histology.** Tissues were fixed in 10% buffered formalin and embedded in paraffin. Multiple sections of the gastrocnemius, soleus, and EDL muscles were obtained and analyzed for adipose tissue content. Staining of the sections was performed with hematoxylin and eosin. For each genotype and muscle type, at least four sections were analyzed. Images were acquired using a BX60 microscope (Olympus) and HV-C20 TV camera (Hitachi, Japan) and were analyzed using Image-Pro Plus 4.0 software.

**Statistical analysis.** Data are expressed as means ± SE. Results were evaluated by repeated-measures ANOVA, ANOVA test, and/or t-test using Statview Software (BrainPower). The difference was considered to be significant if $P \leq 0.05$.

**RESULTS**

Creation and molecular characterization of muscle-specific GLUT4 knockout mice that overexpress GLUT4 selectively in adipose tissue. The cross of mice heterozygous for GLUT4 deficiency selectively in muscle (MCK-Cre$^{+/-}$ Lox$^{-/-}$) to adipose-specific GLUT4 overexpressing mice carrying a floxed GLUT4 allele (AG4Tg Lox$^{-/-}$) yielded six different genotypes. The following three genotypes do not carry the transgene allele: Cre alone (control in our studies), the muscle-GLUT4 heterozygote (MG4Het), and the muscle-GLUT4 knockout (MG4KO). The other three genotypes carry the transgene allele: the adipose-GLUT4 overexpressor (AG4Tg), the adipose-GLUT4 overexpressor muscle-GLUT4 heterozygote (AG4Tg-MG4Het), and the adipose-GLUT4 overexpressor muscle-GLUT4 knockout (AG4Tg-MG4KO). All mice were born at the expected mendelian frequency.

Western blot analysis confirms high-level (>20-fold) overexpression of GLUT4 in adipose tissue from all genotypes that carry the transgene (Fig. 1A), compared with mice that are not carrying the transgene allele (Cre, MG4Het, and MG4KO), consistent with previously published results (12). The level of overexpression is similar in AG4Tg, AG4Tg-MG4Het, and AG4Tg-MG4KO. GLUT4 protein was not detectable in skeletal muscle of MG4KO animals (Fig. 1B). In the muscle-GLUT4 heterozygotes, GLUT4 protein levels in muscle were reduced by ∼50% compared with control mice (data not shown). GLUT4 levels are normal in muscle of AG4Tg mice. A small amount of GLUT4 is detected in muscle of AG4Tg-MG4Het (Fig. 1B), but this is most likely because of contamination of muscle with adipose tissue (Fig. 1, C and D). The lowest band is an inactive form of GLUT4, lacking exon 10 because of Cre-Lox excision of this exon as shown before (39). The in-frame deletion of exon 10 results in a small amount of

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**Fig. 1.** GLUT4 and adipose fatty acid-binding protein (aP2) levels and glucose transport in white adipose tissue (WAT) and skeletal muscle of control (Cre only), adipose GLUT4-overexpressing (AG4Tg) mice, and muscle-GLUT4 knockout (MG4KO) mice with (AG4Tg-MG4KO) or without overexpression of GLUT4 in adipose tissue. GLUT4 protein levels in WAT (A) and in extensor digitorum longus (EDL) muscle (B). C: aP2 protein levels in adipose tissue and skeletal muscle. Tissue lysates were prepared from 2- to 3-mo-old control (C), MG4KO (K), adipose GLUT4 overexpressor (AG4Tg; T), and AG4Tg-MG4KO (TK) mice. GLUT4 (48–50 kDa in control mice) and aP2 (18 kDa) proteins were measured by immunoblotting. Each lane contains protein from a single mouse. Gastroc, gastrocnemius. Data are representative of 3 independent experiments ($n = 12–15$/genotype). D: hematoxylin and eosin (HE) staining of gastrocnemius muscle section from a MG4KO mouse, magnification ×10. Similar fat content was seen in muscles from all genotypes, including controls. The indicated metric bar is 200 μm. E: basal and insulin-stimulated glucose uptake in isolated adipocytes of 2- to 4-mo-old male mice. Results are means ± SE, $n = 6$/genotype. *$P \leq 0.01$ vs. all other genotypes. F: basal and insulin-stimulated glucose transport in soleus muscle. Results are means ± SE, $n = 5–10$/genotype. +$P \leq 0.05$ vs. control and AG4Tg mice. Similar results were obtained for EDL.
a mutated GLUT4 (Fig. 1B) that does not transport glucose (39 and Fig. 1F). As shown in Fig. 1C, Western blot analysis confirms the presence of aP2 protein in EDL, soleus, and gastrocnemius muscles of all genotypes, without differences among genotypes. Because aP2 is an adipose-specific protein, this reflects the fact that it is difficult to dissect the muscle totally free of adipose tissue. Hematoxylin and eosin stains of muscle also confirm the presence of adipose tissue in the dissections (Fig. 1D). This was performed on three different muscle types (EDL, soleus, and gastrocnemius) from all genotypes with similar results.

Glucose uptake ex vivo in white adipose tissue and skeletal muscle of AG4Tg-MG4KO mice. In isolated adipocytes from muscle GLUT4 knockout mice, basal and insulin-stimulated glucose transport were normal. In contrast, in transgenic animals (AG4Tg), basal glucose transport was elevated ~20-fold over basal transport in adipocytes from nontransgenic control mice (Cre) and muscle-GLUT4 knockout mice. Insulin-stimulated glucose transport was increased fourfold over insulin-stimulated transport in nontransgenic control adipocytes (Fig. 1E). This high rate of basal glucose transport reflects increased levels of GLUT4 present at the plasma membrane, even in the absence of insulin resulting from “saturation” of GLUT4 sequestration (10).

We measured glucose transport in soleus muscle, which appeared to have the highest level of adipose infiltration indicated by aP2 amount (Fig. 1C). In soleus muscle of mice overexpressing GLUT4 selectively in adipose tissue (AG4Tg), basal and insulin-stimulated glucose transport are normal, as expected (Fig. 1F). Also as expected, in soleus muscle of muscle-G4KO animals, the basal glucose transport is decreased 83%, and the response to insulin is absent. Similar results were obtained in EDL muscle (data not shown), as observed previously (39). In addition, basal glucose transport ex vivo in soleus muscle of AG4Tg-MG4KO animals was markedly reduced compared with control and AG4Tg littermates, and the insulin-stimulated glucose uptake in these muscles was reduced by ~80% compared with both control and AG4Tg animals. Both the basal and the insulin-stimulated glucose uptake ex vivo in muscle of the AG4Tg-MG4KO mice are less than that observed in the basal state of the control animals (Fig. 1F). Also, in vivo glucose transport in gastrocnemius and EDL muscle of AG4Tg-MG4KO is markedly reduced compared with control (see Fig. 4C).

Growth curves and body composition. Growth curves in female animals show that muscle-G4KO animals are significantly lighter than all the other genotypes through at least 20 wk of age (Fig. 2A). Even at 3 wk of age, MG4KO mice already weighed less than controls (11.1 ± 0.5 g compared with 13.4 ± 0.4 g for controls, P = 0.05). The weight of male muscle-G4KO mice at 3 wk is not different from the other genotypes, but it is lower compared with control starting at week 4 through week 20 (data not shown). This differs from our previous report of normal body weight in muscle-G4KO mice (39). The earlier onset of leanness in this study may be because of 1) differences in the genetic admixture of the two groups resulting from breeding the AG4Tg mice, which are on an inbred FVB background into the MG4KO line; 2) a change in the composition of the new diet, and 3) differences in the ambient temperature in the animal facility we used for this study compared with the previous one (39). Growth curves for the muscle-GLUT4 heterozygous animals show an intermediate phenotype (data not shown). Mice with Tg allele (AG4Tg and AG4Tg-MG4KO) are slightly heavier than controls (Fig. 2A). We previously published that the high-level overexpression of GLUT4 is maintained from 7 wk to at least 1 yr of age (15, 32). Furthermore, the effect of the Tg allele on body weight was sustained throughout this period in mice on an inbred FVB background (15, 32). Throughout the entire 6 mo in the current study, overexpression of GLUT4 in adipose tissue “rescues” the decreased weight in mice lacking GLUT4 in muscle (Fig. 2A).

The increase in weight in mice with the Tg allele is attributable to adipose tissue, as determined by DEXA (Fig. 2, B and C). The decrease in body weight in MG4KO mice is the result of decreased fat mass compared with all other groups. Mice that overexpress GLUT4 specifically in adipose tissue (AG4Tg or AG4Tg-MG4KO) have significantly more body fat than those that do not overexpress GLUT4 in adipose tissue (Fig. 2, B and C). Therefore, expression of GLUT4 in adipose tissue reverses the leanness in MG4KO mice. There was a similar increase in body fat in AG4Tg-MG4KO compared with MG4KO (data not shown). There is no change in cell size in mice overexpressing GLUT4 in adipose tissue compared with controls (10, 32).

Overexpression of GLUT4 selectively in adipose tissue restores glucose tolerance in muscle GLUT4 KO mice. At both 2 and 12 mo of age, AG4Tg and AG4Tg-MG4KO mice have fasting glucose levels that are 30% lower than control mice (Fig. 3A). Glucose levels at all times during the glucose
tolerance test are also lower than controls. Muscle-GLUT4 KO mice have higher fasting glucose levels (96 \pm 3 \text{ vs. } 79 \pm 7 \text{ mg/dl in controls}) and glucose intolerance as early as 2 mo of age that persists to 12 mo (Fig. 3A). Overexpression of GLUT4 in adipose tissue of muscle-GLUT4 KO mice not only rescues their glucose intolerance but lowers their fasting glucose (55 \pm 4 \text{ vs. } 76 \pm 7 \text{ mg/dl in controls}) and glucose excursion below control levels. Similar results were obtained at 6 mo of age (data not shown). MG4Het and AG4Tg-MG4Het mice showed an intermediate phenotype compared with controls and muscle-GLUT4 KO mice (data not shown). Figure 3A shows results for females; similar results were seen in males (data not shown).

**Plasma glucose profile after removal of food and insulin tolerance test.** When food was removed at 8:30 A.M. from female mice, plasma glucose concentrations in control and MG4KO fell slowly, reaching a 30% decrease over 7 h. In contrast, in AG4Tg and AG4Tg-MG4KO, glucose levels fell almost 70% during the 7-h period (Fig. 3B), and blood glucose was as low as 40 mg/dl after an overnight fast (Fig. 3A, 12 mo old).

After removal of food for 4 h, an insulin (0.75 U/kg body wt) tolerance test (ITT) was performed (Fig. 3C). Insulin resistance was evident in muscle-GLUT4 KO mice as shown previously (39). After insulin injection, glucose levels did not decrease significantly in female (from 113 \pm 4 \text{ to } 94 \pm 6 \text{ mg/dl}) or male MG4KO mice (data not shown) compared with control mice (from 86 \pm 10 \text{ to } 48 \pm 2 \text{ mg/dl}). Both AG4Tg (48 \pm 7 \text{ fell to } 26 \pm 1 \text{ mg/dl}) and AG4Tg-MG4KO (51 \pm 2 \text{ to } 37 \pm 3 \text{ mg/dl}) were so low at the beginning of the ITT that there was very little margin for further lowering of glucose. Preliminary data show an elevation of plasma glucagon in hypoglycemic fasted AG4Tg mice, and it is likely that counterregulatory hormones prevent a further fall in blood glucose. Of note, the initial blood glucose values in Fig. 3C are comparable to the 12:00 P.M. values in Fig. 3B, i.e., measured 4 h after food removal. The differences in the absolute values between Fig. 3, B and C, are due, at least in part, to the fact that Fig. 3B shows plasma glucose, whereas Fig. 3C shows whole blood glucose. These are known to differ by at least 14%.

**Serum levels of insulin and adipocyte-secreted molecules.** Metabolic parameters were measured in both female (Table 1) and male (Table 2 and data not shown) mice at several different ages between 2 and 12 mo of age. Similar effects were seen for all parameters in both genders. Table 1 shows body weights for the female mice at 2–3 mo of age in which the adipocyte-secreted molecules were measured. Consistent with data in Fig. 2, AG4Tg and AG4Tg-MG4KO mice were significantly heavier than control and MG4KO mice, and MG4KO mice were slightly lighter than all other groups. Fasting and fed blood glucose were higher in MG4KO mice than in all other genotypes, and fasting glucose was lower in AG4Tg and AG4Tg-MG4KO mice compared with controls, consistent with Fig. 3. Fasted insulin levels tended to be lower in AG4Tg and AG4Tg-MG4KO mice compared with control and MG4KO mice (Table 1). Fed insulin levels were lower in female AG4Tg mice compared with MG4KO mice at 2 mo of age. FFA in both the fasting and fed states were elevated in AG4Tg and Ag4Tg-MG4KO mice (Table 1). Fed insulin levels were lower in female AG4Tg mice compared with MG4KO mice at 2 mo of age. FFA in both the fasting and fed states were elevated in AG4Tg and Ag4Tg-MG4KO mice.

**Fig. 3.** Glucose homeostasis in control, AG4Tg, and MG4KO mice with or without overexpression of GLUT4 in adipose tissue. A: glucose tolerance tests (2 mg/g ip) were performed after an overnight fast (15 h) in 2- and 12-mo-old female mice. Control (○), MG4KO (▲), AG4Tg (■), and AG4Tg-MG4KO (●) mice, n = 10–13/group. B: plasma glucose monitoring hourly for 7 h in 3-mo-old female mice after food removal at 8:30 A.M., n = 4–9/group. **P \leq 0.05 vs. mice without the Tg allele for all points after 8:00 A.M. C: insulin tolerance tests were performed after removal of food for 4 h. Mice were injected ip with 0.75 U/kg body wt of human insulin, n = 10–13/group. Results are means \pm SE. *P \leq 0.05 for all genotypes vs. all others. †P \leq 0.05 vs. MG4KO and AG4Tg.
MG4KO mice compared with all other genotypes, and overexpression of GLUT4 in adipose tissue reversed this elevation (Table 1). The increase in resistin might contribute to the insulin-resistant state in MG4KO mice. We investigated this possibility with signaling studies (see below).

In vivo glucose metabolism and 2-DG uptake in individual tissues during a hyperinsulinemic-euglycemic clamp. Euglycemic-hyperinsulinemic clamp studies were performed on both male and female mice, and similar effects of MG4KO and AG4Tg were seen in both genders. Table 2 shows data for the male mice undergoing clamp studies. In these studies, basal refers to after food removal for 3–5 h before the beginning of the clamp. Plasma glucose before the clamp was elevated in AG4Tg mice compared with all other genotypes, and it was lower in AG4Tg and AG4Tg-MG4KO compared with mice without the Tg allele. Serum insulin levels before the clamp tended to be elevated in MG4KO mice. Previously, we reported a large range in insulin values in MG4KO mice that are on an outbred background (19, 39). This makes it difficult to see a statistically significant difference in the mean value, although the distribution of values differs between MG4KO mice and controls. We previously found that the low range of insulin values was eliminated in MG4KO mice, and 30% of MG4KO mice had insulin values higher than any of the control mice (19, 39).

Insulin levels during the clamp were similar in all groups of mice (Table 2). Serum FFA levels were elevated in male AG4Tg and AG4Tg-MG4KO compared with control and MG4KO mice before the clamp (3–5 h after food removal; Table 2). In control mice, insulin lowered FFA from 0.78 ± 0.05 to 0.40 ± 0.06 mM. Although FFA levels in AG4Tg and AG4Tg-MG4KO mice were reduced by insulin infusion during the clamp, they remained elevated compared with controls after insulin infusion (Table 2). In MG4KO animals, insulin infusion did not lower FFA levels, indicating insulin resistance in adipose tissue. Overexpression of GLUT4 in adipose tissue of MG4KO mice raises FFA levels above control and reverses the defect in insulin-induced suppression of FFA in MG4KO mice.

Table 1. Metabolic parameters in female control (Cre), MG4KO, AG4Tg, and AG4Tg-MG4KO mice

<table>
<thead>
<tr>
<th>Genotypes, units</th>
<th>Control</th>
<th>MG4KO</th>
<th>AG4Tg</th>
<th>AG4Tg-MG4KO</th>
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<tr>
<td>Body weight, g</td>
<td>24.4 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.2 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.2 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Fasted blood glucose, mg/dl</td>
<td>69 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Fed blood glucose, mg/dl</td>
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<td>127 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>112 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>112 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Fasted insulin, pg/ml</td>
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<td>430 ± 110&lt;sup&gt;a&lt;/sup&gt;</td>
<td>287 ± 44&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Fed insulin, pg/ml</td>
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<td>740 ± 53&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>652 ± 77&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Fed FFA, mM</td>
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<td>1.01 ± 1.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.50 ± 1.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Fed FFA, mM</td>
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<td>Fed leptin, ng/ml</td>
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<td>1.70 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.50 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.40 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fed resistin, ng/ml</td>
<td>4.36 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.84 ± 0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.43 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.91 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fed adiponectin, μg/ml</td>
<td>30.5 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.8 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.8 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.1 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–25/genotype. MG4KO, muscle-GLUT4 knockout; AG4Tg, adipose GLUT4 transgenic; FFA, free fatty acid; fed, animals bled between 8:00 and 9:00 A.M.; fasted, animals fasted overnight. Mice were 2–3 mo old for all measurements except resistin (measurement at 4–6 mo old). Glucose values were determined on whole blood. For each parameter, values with different superscripted letters are different at P < 0.05.

Table 2. Metabolic parameters in male control (Cre), MG4KO, AG4Tg, and AG4Tg-MG4KO mice undergoing euglycemic-hyperinsulinemic clamp

<table>
<thead>
<tr>
<th>Genotype, units</th>
<th>Control</th>
<th>MG4KO</th>
<th>AG4Tg</th>
<th>AG4Tg-MG4KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>33.0 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.0 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.0 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.0 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Basal plasma glucose, mg/dl</td>
<td>141 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>195 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73 ± 13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clamp plasma glucose, mg/dl</td>
<td>137 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>123 ± 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>124 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>136 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Basal insulin, pg/ml</td>
<td>364 ± 66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>530 ± 197&lt;sup&gt;a&lt;/sup&gt;</td>
<td>237 ± 86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>348 ± 85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clamp insulin, pg/ml</td>
<td>3,166 ± 257&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3,591 ± 590&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2,848 ± 199&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2,582 ± 203&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Basal FFA, mM</td>
<td>0.78 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.55 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.54 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clamp FFA, mM</td>
<td>0.40 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

Values are means ± SE. Mice were 3 mo old. Glucose values were determined on plasma. Basal indicates before initiation of the clamp and was 3–5 h after food removal at 8:00 A.M. Clamp refers to hyperinsulinemic (2.5 mU·kg<sup>–1</sup>·min<sup>–1</sup>)-euglycemic clamp. Mice were unrestrained during the clamp. *P < 0.05 vs. basal FFA for the same genotype. For each parameter, values with different superscripted letters are different at P < 0.05.
control mice. In AG4Tg-MG4KO mice, it is also markedly reduced, although 2-DG uptake is slightly higher in gastrocnemius of AG4Tg-MG4KO mice compared with MG4KO mice (Fig. 4C). Similarly, previously when blood glucose was normalized in MG4KO mice with phloridzin treatment, glucose transport in muscle increased threefold (17). Importantly, in the current study, insulin-stimulated glucose transport in gastrocnemius muscle of AG4Tg-MG4KO mice is still less than half the rate in the gastrocnemius of control mice. Furthermore, there is less tendency for an increase in AG4Tg-MG4KO compared with MG4KO in EDL muscle, and this is not statistically significant. Although we cannot eliminate the possibility that a small increase in glucose transport in some muscles of AG4Tg-MG4KO mice compared with MG4KO mice might contribute to the reversal of insulin resistance, it is unlikely to explain the majority of the effect. Importantly, the improvement in glucose homeostasis in AG4Tg-MG4KO mice occurs without restoring glucose transport in muscle to normal levels or even to 50% of normal levels.

Adipose-specific overexpression of GLUT4 (AG4Tg and AG4Tg-MG4KO) caused an 80% to twofold increase in the insulin-stimulated 2-DG uptake in adipose tissue in vivo compared with control and MG4KO mice (Fig. 4D). Because whole body fat mass is increased in AG4Tg and AG4Tg-MG4KO mice, the actual amount of glucose going in the adipose organ is increased even more than twofold. Insulin-stimulated 2-DG uptake in white adipose tissue (WAT) of MG4KO mice in vivo was normal. Previously, we found an increase in 2-DG uptake in WAT of MG4KO mice at 10 wk of age but by 20–21 wk of age, 2-DG was decreased in WAT (17).

**Impact of adipose-specific overexpression of GLUT4 on liver glucose flux and lipid metabolism.** MG4KO animals are only mildly diabetic under ambient conditions, even though glucose uptake in muscle is markedly reduced (Fig. 4C), and serum insulin levels are only modestly increased (Table 1). We previously found increased glucose flux in the liver in MG4KO mice (39) and mice lacking GLUT4 in both muscle and adipocytes (19), which could lessen the severity of hyperglycemia. Consistent with this, in the current study, glucokinase protein levels are 50% higher in MG4KO mice compared with control and 73% higher compared with AG4Tg mice (Fig. 5A), reflecting increased glucose uptake by liver in MG4KO mice (19). Because insulin action on hepatic glucose production is a completely separate process from hepatic glucose uptake and both can also be regulated by fatty acids and glucose as well as by insulin, it is possible to increase both parameters when metabolism is dysregulated. This elevation of glucokinase protein was restored to normal in AG4Tg-MG4KO mice (Fig. 5A).

We previously showed that mice lacking GLUT4 in both muscle and adipocytes clear lipids more effectively from blood than control mice using an oral triglyceride loading test (19). Because AG4Tg mice have markedly reduced ability to utilize glucose in muscle (Figs. 1F and 4C), we hypothesized that they might use more lipid. In AG4Tg-MG4KO, FFA levels were greater than control and MG4KO 4 h after food was removed (Fig. 5B, time 0), consistent with data in Table 1 (female, fasting) and Table 2 (male, 3–5 h after food removal). Although serum FFA levels rose during the first 2–3 h after the oral lipid gavage in all the genotypes studied (Fig. 5B), the clearance of FFA was much faster in MG4KO mice than in control and transgenic littermates. Both the lower baseline FFA levels and the increased clearance rate were reversed in AG4Tg-MG4KO mice. Initial serum triglyceride levels for transgenic animals of both genotypes showed a serum triglyceride peak between 2 and 3 h, MG4KO mice had a flat triglyceride response to lipid gavage, indicating a much faster triglyceride clearance rate (Fig. 5C). We also carried out these studies with more than three times the amount of lipid in the
gavage. Also, with this higher dose, lipid clearance was markedly enhanced in MG4KO compared with all other groups, and this effect was reversed with adipose-GLUT4 overexpression in muscle-GLUT4 KO mice (data not shown).

Signaling through the AMPK-ACC pathway in liver and muscle. Because recent data suggest that elevated levels of resistin may play a role in regulating AMPK phosphorylation in liver in the high-fat-fed state (24), we measured AMPK and ACC phosphorylation and activity in our mice. AMPK phosphorylates ACC and inhibits its activity. The amount of α2-AMPK subunit protein and its phosphorylation and both α1-

and α2-AMPK activities in the liver of all genotypes in our study are not changed. As shown in Fig. 6, ACC protein (Fig. 6A) and phosphorylation (Fig. 6B) levels are elevated in liver of MG4KO compared with control mice. Overexpression of GLUT4 in adipose tissue (AG4Tg-MG4KO) reverses this effect. The increase in total ACC in MG4KO seems to be the main determinant of increased phospho-ACC, since phospho-ACC corrected for total ACC is no longer significantly increased in MG4KO mice (Fig. 6C). This is consistent with the lack of change in ACC activity in liver of any of the genotypes (Fig. 6D).

We also measured AMPK and ACC protein, phosphorylation, and activity in muscle. In both soleus and EDL muscles, similar to liver, there is no difference in either protein amount or phosphorylation of AMPK (data not shown). Although α2-AMPK activity tended to be increased in EDL of MG4KO mice, this did not reach statistical significance. On the other hand, total ACC protein is decreased ~45% in the EDL of MG4KO (69 ± 6 arbitrary units) compared with control mice (118 ± 17; P ≤ 0.05), and tends to be decreased (~20%) in soleus of MG4KO mice. Thus absence of GLUT4 in muscle has opposite effects on total ACC protein levels in muscle and liver, and this does not appear to be regulated at the level of AMPK. There were no changes in AMPK or ACC protein or activity in muscle of AG4Tg or AG4Tg-MG4KO mice.

DISCUSSION

This study and our previous work (39) show that muscle GLUT4 knockout mice, both homozygous and heterozygous, are insulin resistant, glucose intolerant, and have fasting hyperglycemia. We previously showed that overexpression of GLUT4 selectively in adipose tissue results in enhanced insulin sensitivity and glucose tolerance (32). Therefore, we hypothesized that overexpression of GLUT4 in adipose tissue might reverse insulin resistance because of the lack of GLUT4 in muscle. We pursued these studies since in humans with obesity...
and/or type 2 diabetes (8, 31), as well as in family members of diabetic subjects who have a high risk for type 2 diabetes (9). GLUT4 is downregulated selectively in adipose tissue while insulin resistance is also present in muscle. Here we find that overexpression of GLUT4 in adipose tissue not only rescues the insulin resistance resulting from impaired glucose transport in muscle but also lowers fasting glucose and glucose excursion below control levels, enhancing glucose tolerance so that it is better than in control mice. This insulin sensitivity occurs, surprisingly, in the presence of modestly increased fat mass and serum FFA concentrations and decreased serum adiponectin levels in mice overexpressing GLUT4 in adipose tissue.

There are several potential mechanisms for increased insulin sensitivity in the setting of increased fat mass. First, small adipocytes are in most cases more sensitive to insulin than large ones in terms of glucose transport and metabolism (13), and an increase in small adipocytes provides more surface area for glucose uptake. Small adipocytes also tend to confer more insulin sensitivity systemically, which may be due, in part, to a more favorable pattern of adipokine secretion than with large adipocytes (12). Consistent with this, insulin-sensitizing drugs in the thiazolidinedione class decrease adipocyte size while maintaining or increasing total body fat mass (27). These insulin-sensitizing agents result in decreased resistin and increased adiponectin secretion, which may contribute to their insulin-sensitizing effects. The mild obesity in AG4Tg mice is the result of adipocyte hyperplasia without an increase in adipocyte size (10, 32, 34). Thus the increased number of small adipocytes present in mice overexpressing GLUT4 in adipocytes (10, 32, 34) might be expected to cause a relative increase in insulin sensitivity.

Surprisingly, in AG4Tg and AG4Tg-MG4KO mice, serum adiponectin is low, which is unusual for an insulin-sensitive state. Plasma adiponectin concentrations correlate with insulin sensitivity in most models (28, 36). Adiponectin levels are low in most insulin-resistant states, and administration of recombinant adiponectin to diabetic rats and to adiponectin knockout mice improves insulin sensitivity (20, 37). Our data suggest that other factors can compensate for decreased adiponectin levels in terms of systemic insulin sensitivity. Leptin levels are modestly elevated in AG4Tg and AG4Tg-MG4KO mice, consistent with their increased fat mass, and, potentially, this could contribute to increased insulin sensitivity.

Evidence is mounting for the concept that the liver plays a potentially more important role than muscle in glucose homeostasis, at least in the mouse (18). Because in the absence of GLUT4 it is not possible to restore glucose uptake in the muscle to normal (Figs. 1F and 4C), the restored insulin sensitivity in AG4Tg-MG4KO mice might also reflect alterations in hepatic glucose fluxes (21). The defect in insulin action on hepatic glucose production in MG4KO mice is reversed by overexpression of GLUT4 in adipose tissue (Fig. 4B). In addition, reversal of the elevation of glucokinase seen in liver of MG4KO by adipose GLUT4 overexpression most likely indicates a change in glucose flux in the liver. We previously showed in mice deficient in GLUT4 in both muscle and adipocytes that glucose uptake by the liver is increased (19). Data in the current manuscript suggest that, with increased glucose uptake in adipocytes, even though glucose transport in muscle is markedly reduced, a compensatory increase in hepatic glucose uptake does not occur. In MG4KO mice, similar to mice lacking GLUT4 in both muscle and adipose tissue (19), utilization of fatty acids and triglycerides is increased (Fig. 5, B and C), and this effect is also reversed by overexpression of GLUT4 in fat.

Resistin, an adipocyte-secreted molecule, may play a role in the regulation of glucose homeostasis (33) and hepatic glucose production (29). The elevation of resistin levels in MG4KO mice could be expected to increase hepatic glucose production and impair insulin action to suppress hepatic glucose production (29). Data in Fig. 4B are consistent with this although there are no published data on the effect of such a mild elevation of resistin. This elevation of resistin is also reversed by overexpression of GLUT4 in adipose tissue, and this may be at least part of the mechanisms for reversal of hepatic insulin resistance. Recent studies suggest that resistin inhibits AMPK phosphorylation in the liver (4, 24). We found no changes in AMPK phosphorylation or activity in liver of MG4KO mice in spite of modestly increased resistin levels. Total protein expression of the key lipogenic enzyme, ACC, was increased 60% in liver of MG4KO mice compared with control littermates, and this effect was totally reversed by overexpression of GLUT4 in adipose tissue of these mice (AG4Tg-MG4KO; Fig. 6A). However, the inactive, i.e., phosphorylated form, of ACC is increased to a similar extent (Fig. 6, B and C), resulting in no change in ACC activity (Fig. 6D). Thus the normalization of resistin levels could still be important, although the mechanism by which it improves insulin sensitivity does not appear to be through AMPK-ACC signaling.

Another consideration is whether increased glucose transport in adipose tissue can account for the increased systemic glucose disposal in AG4Tg-MG4KO mice. In vivo glucose transport in WAT of AG4Tg and AG4Tg-MG4KO mice is approximately doubled compared with control and MG4KO mice. With the expanded fat mass, the increase in glucose transported in the adipose organ is probably even greater. Glucose transport in adipocytes of AG4Tg mice is increased over control levels to a greater extent ex vivo than in vivo, most likely because of the fact that the ex vivo study is performed at tracer glucose concentration at which glucose transport is completely rate limiting for glucose metabolism (34). Nevertheless, the increase in glucose transport in WAT may contribute to the restoration of glucose homeostasis. The systemic effect may not be large, however, since there is no increase in GINF or R₄ in AG4Tg mice compared with controls. The 20-fold overexpression of GLUT4 in adipose tissue in this study may seem unphysiological. However, we originally also characterized another line of mice with lower-level overexpression of GLUT4 in adipose tissue, and they showed a similar phenotype (32).

In summary, this study demonstrates that markedly enhancing glucose transport in adipose tissue can overcome impaired insulin-stimulated glucose uptake in muscle even in the setting of increased adiposity. Thus mild obesity can be dissociated from insulin resistance when glucose transport is high in adipose tissue. Improved glucose homeostasis can also occur with increased FFA levels and low adiponectin. These results reinforce the central role for adipose tissue in regulating insulin sensitivity and energy balance. The potential relevance of this model to humans is that expression of GLUT4 is selectively reduced in adipocytes of obese and type 2 diabetic individuals (31) and in first-degree relatives of people with type 2 diabetes.
who are healthy but have a genetic predisposition for type 2 diabetes (8, 9). Because GLUT4 is downregulated selectively in adipose tissue and not in muscle in these individuals and glucose transport is impaired in their muscle, restoring GLUT4 levels and glucose transport in adipose tissue might improve their insulin sensitivity, although it could also worsen their obesity.

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