Amelioration of Insulin Resistance But Not Hyperinsulinemia in Obese Mice Overexpressing GLUT4 Selectively in Skeletal Muscle

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The effects of gold-thioglucose (GTG) treatment were examined in mice overexpressing GLUT4 selectively in skeletal muscle (MLC-GLUT4 mice) and in age-matched controls. Groups of MLC-GLUT4 and control mice were injected with GTG or saline at 5 weeks of age. At 12 weeks following the injections, GTG-treated control mice exhibited a 35% increase in body weight versus saline-treated controls. Similarly, a 30% increase in body weight was observed in GTG-treated MLC-GLUT4 mice compared with saline-treated MLC-GLUT4 mice 12 weeks after the injections. In saline-treated lean MLC-GLUT4 and control mice, intraperitoneal injection of insulin decreased blood glucose in 1 hour by 63% and 38%, respectively. Insulin also decreased blood glucose by 40% in GTG-treated obese MLC-GLUT4 mice after 1 hour. However, insulin did not reduce blood glucose levels in GTG-treated obese control mice. The ability of insulin to clear blood glucose in GTG-treated obese MLC-GLUT4 mice is associated with increased skeletal muscle GLUT4 content and white adipose tissue (WAT) GLUT4 content as compared with GTG-treated obese controls. However, fasting blood glucose levels in GTG-treated obese MLC-GLUT4 and control mice were elevated by approximately 30% compared with saline-treated groups. Lastly, although GTG-treated obese MLC-GLUT4 mice exhibited improved glucose clearance in response to insulin, they nevertheless remained as hyperinsulinemic as GTG-treated obese control mice. These results suggest that genetic overexpression of GLUT4 in skeletal muscle may ameliorate the development of insulin resistance associated with obesity but cannot restore normal glucose and insulin levels.

SKELETAL MUSCLE plays a major role in the maintenance of glucose homeostasis since it is the major site of glucose disposal under insulin-stimulated conditions.[1] Impaired skeletal muscle glucose utilization under insulin action is one of the most important abnormalities during the pathological development of type 2 diabetes mellitus.[2,3] In particular, impairment of skeletal muscle glucose transport in response to insulin is thought to contribute significantly to muscle insulin resistance in both humans and animals with type 2 diabetes mellitus.[4-7]

Glucose transporter 4 (GLUT4) is the major facilitative glucose transporter isoform expressed in skeletal muscle.[8,9] In skeletal muscle, GLUT4 is translocated from intracellular stores to the sarcolemma and T tubules upon stimulation by insulin, ischemia, or exercise.[10-13] Recently, our group and others have generated transgenic mice that overexpress GLUT4 either selectively in skeletal muscle[14,15] or in all tissues that normally express GLUT4.[16-18] Studies with these transgenic mice indicate that overexpression of GLUT4 can increase glucose transport and improve whole-body insulin action.[17,19,20] Overexpression of GLUT4 has been shown to ameliorate impaired whole-body glucose homeostasis in mice with a homozygous mutation in the leptin receptor (*db/db* mice) or rendered diabetic by streptozotocin treatment.[15,21] It has been proposed that the stimulation of muscle glucose uptake in type 2 diabetic patients may have beneficial effects.[3] Studies with GLUT4 transgenic mice suggest that a new mode of therapy based on increased GLUT4 expression and/or activity may prove effective in restoring normal glucose homeostasis in type 2 diabetes.[14,15,17,19-21]

Peripheral insulin resistance is also associated with both human obesity[22] and animal models of obesity.[23] In mice, gold-thioglucose (GTG) injection causes a lesion in the ventromedial hypothalamus resulting in hyperphagia, increased body weight gain, hyperglycemia, and hyperinsulinemia.[23] Impaired skeletal muscle glucose uptake has been demonstrated to be part of the disrupted glucose homeostasis in GTG-induced obesity.[6] However, expression levels of GLUT4 in skeletal muscle of GTG-treated mice remain normal.[24] In rodents with hypothalamic lesions, insulin resistance develops secondary to weight gain and hyperinsulinemia.[23] The cause of hyperinsulinemia in GTG-induced obesity is presently unclear. It remains to be determined whether it is due to insulin resistance, increased adipose tissue mass, disruption of the neuroendocrine-pancreas axis, or a combination of these factors.

We have previously described a line of transgenic mice that overexpress GLUT4 selectively in fast-twitch skeletal muscle using the myosin light-chain 1 promoter (MLC-GLUT4 mice).[14,25] These MLC-GLUT4 mice exhibited increased 2-deoxyglucose uptake in fast-twitch skeletal muscle, increased insulin-stimulated whole-body glucose utilization, and improved glucose homeostasis.[14] To test the hypothesis that increased GLUT4 expression in skeletal muscle can ameliorate the onset of obesity-induced insulin resistance, we treated both control and MLC-GLUT4 mice with GTG to induce obesity. MLC-GLUT4 mice were as susceptible as control mice to the development of obesity following GTG treatment, despite a sustained increase in skeletal muscle GLUT4 content. In control mice, GTG-induced obesity is accompanied by the development of impaired insulin action as assessed by an insulin tolerance test. However, the development of impaired insulin action associated with GTG-induced obesity was prevented in MLC-GLUT4 mice, uncoupling obesity from insulin resistance. These results indicate that expression of the MLC-GLUT4 transgene in skeletal muscle can ameliorate the development of

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obesity-associated insulin resistance following GTG treatment. Furthermore, the persistence of hyperinsulinemia in GTG-treated obese MLC-GLUT4 mice suggests that insulin resistance does not likely play a predominant role in causing hyperinsulinemia in GTG-induced obesity.

MATERIALS AND METHODS

Animals

The generation and identification of MLC-GLUT4 mice have been described previously.[14] Transgenic mice were maintained in a CBA/C57B16 hybrid background. Age-matched control mice were the offspring of matings between CBA males and C57B16 females. Male mice were used in all studies. Animals were fed ad libitum and maintained in a murine hepatitis virus–free barrier facility on a 12-hour light/dark cycle. All protocols were approved by the Animal Care and Use Committee of Albert Einstein College of Medicine in accordance with the Public Health Service Animal Welfare Policy.

GTG Treatment

GTG (Fluka, Ronkonkoma, NY) was dissolved in sterile saline (100 mg/3 mL) immediately before injection. GTG was injected intraperitoneally at 1.15 mg/g body weight between 11:00 AM and 12:00 noon under ad libitum–fed conditions with a 26-gauge needle. Another group of mice received sterile saline injections. Following GTG or saline injection, the mice were weighed approximately once per week to monitor the course of weight gain. Experiments were performed on mice at 26 to 28 weeks of age, when obesity has already reached a plateau.

Immunoblot Analysis of GLUT4

Hindlimb skeletal muscle, white adipose tissue (WAT), and heart were homogenized in TES buffer (100 mmol/L Tris, pH 7.6, 0.2 mmol/L EDTA, and 255 mmol/L sucrose) supplemented with a protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) containing 1 mmol/L 4-(2-aminoethyl)-benzenesulfonyl-fluoride, 0.3 μmol/L aprotinin, 1 μmol/L pepstatin, and 1 μmol/L leupeptin. WAT homogenates were centrifuged at 4,000 × *g* for 15 minutes to separate triglyceride from the aqueous phase. Only the homogenate fat-free aqueous phase was used in analyses. The amount of protein in the homogenate was determined by bicinchoninic acid assay (Pierce, Rockford, IL) using bovine serum albumin as a reference standard. Immunoblot analyses of homogenates were performed as previously described.[14,26]

Insulin Tolerance Test

Following a 6-hour fast, porcine insulin was injected intraperitoneally (1.8 U/kg body weight) and blood was withdrawn from the retro-orbital sinus at 0, 15, 30, and 60 minutes following insulin injection. Blood glucose levels were measured using the One Touch II glucometer system (Lifescan, Milpitas, CA). After the last sampling, an intraperitoneal injection of glucose solution (1 mg/g body weight in saline) was administered to prevent hypoglycemia.

Plasma Metabolites and Hormones

Blood was drawn from the retro-orbital sinus using a heparinized microcapillary tube and quickly vortexed in a microcentrifuge to obtain plasma. Plasma insulin levels were measured using a rat insulin kit (Linco, St Louis, MO). Plasma glucose and lactate levels were determined using Trinder oxidase kits (Sigma Diagnostics, St Louis, MO). Plasma free fatty acid (FFA) levels were measured using a kit from Amano (Richmond, VA) with oleic acid as a standard.

Statistical Analysis

Data are presented as the mean ± SE of multiple determinations. Statistical significance was evaluated by ANOVA using Fisher's paired least-significant difference (PLSD) test for post hoc analysis. Significance was accepted at a P level less than .05.

RESULTS

Development of Obesity Following GTG Administration

GTG (1.15 mg/g) was injected intraperitoneally into six 5-week-old male control (CBA/C57B16 F1) and 5 age- and sex-matched MLC-GLUT4 mice under postprandial conditions. A similar number of male control and MLC-GLUT4 mice were injected with saline and served as lean controls. A percentage of control (5 of 6) and MLC-GLUT4 (4 of 5) mice developed gross obesity following GTG treatment. The course of obesity development is shown in Fig 1. The development of obesity in GTG-treated MLC-GLUT4 mice was less rapid than in similarly treated controls. The small difference in the rate of weight gain between obese MLC-GLUT4 and control groups resulted in a mere 7% decrease in the body weight of obese MLC-GLUT4 mice compared to obese controls at 17 weeks of age. At 28 weeks of age, GTG-treated MLC-GLUT4 mice weighed nearly as much as the similarly treated controls. Both control and MLC-GLUT4 mice entered a rapid phase of weight gain almost immediately after GTG injection. This dynamic phase of weight gain ended approximately 1 month after treatment, when the rate of weight gain started to decrease to the same level as saline-treated groups. No difference in the pattern of weight gain was observed between saline-injected lean MLC-GLUT4 and control mice.

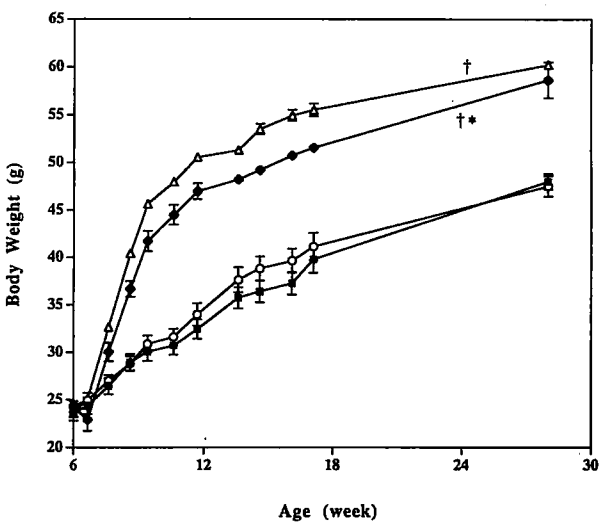

Fig 1. Growth curve for lean control (○), lean MLC-GLUT4 (■), obese control (△), and obese MLC-GLUT4 (◆) mice following injection of saline or GTG. The number of observations in each group is 4 for obese MLC-GLUT4 mice and 5 for each of the lean MLC-GLUT4, lean control, and obese control groups. †$P < .0001$ by ANOVA for repeated measures using Fisher's PLSD post hoc analysis for differences between each of the lean and obese groups. The course of weight gain is significantly different between obese control and obese MLC-GLUT4 groups (*$P < .03$).

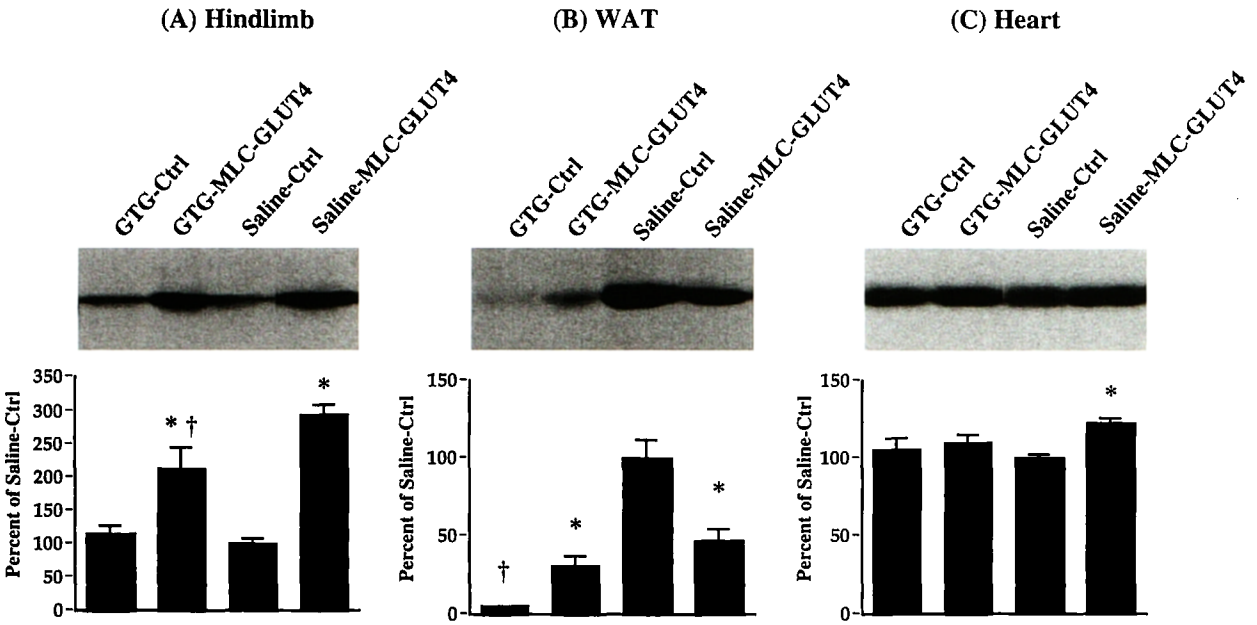

Fig 2. Immunoblot analysis of GLUT4 in the hindlimb skeletal muscle, WAT, and heart of saline- and GTG-treated control and MLC-GLUT4 mice. The number of observations in each group is 4 for obese MLC-GLUT4 mice and 5 for each of the lean MLC-GLUT4, lean control, and obese control groups; 50 μg protein was loaded in each lane. *$P < .05$ for similarly treated MLC-GLUT4 *v* control groups by ANOVA for repeated measures using Fisher's PLSD post hoc analysis. †$P < .05$ for saline- *v* GTG-treated groups.

Immunoblot Analysis

Western blot analysis was performed to determine the amount of immunoreactive GLUT4 in the hindlimb skeletal muscle, WAT, and heart of MLC-GLUT4 transgenic and control mice treated with GTG or saline. Results of the immunoblot analysis are shown in Fig 2. No difference in skeletal muscle GLUT4 content was observed between control mice injected with saline or GTG. Compared with saline-injected lean controls, similarly treated lean MLC-GLUT4 mice exhibited a 194% increase ($P < .0001$) in skeletal muscle GLUT4. A small decrease (28%, $P < .01$) in GLUT4 content was detected in skeletal muscle of GTG-treated obese MLC-GLUT4 mice compared with saline-treated lean MLC-GLUT4 mice. The amount of immunoreactive GLUT4 in GTG-treated MLC-GLUT4 skeletal muscle nevertheless remained elevated by 84% ($P < .005$) over that found in GTG-treated obese control mice. A 94% decrease ($P < .0001$) in WAT GLUT4 content was observed in GTG-treated obese control mice versus saline-treated lean control mice. The downregulation of WAT GLUT4 in the obese control group following GTG treatment was not observed in the GTG-treated obese MLC-GLUT4 group. WAT GLUT4 content in GTG-treated obese control mice was only 19% of that observed in similarly treated obese MLC-GLUT4 mice ($P < .05$). Compared with the saline-treated control group, the similarly treated MLC-GLUT4 group exhibited a 53% decrease ($P < .0005$) in immunoreactive WAT GLUT4. With the exception of a small increase in the saline-treated MLC-GLUT4 group compared with the saline- or GTG-treated control groups (23% and 16%, respectively, $P < .03$), GLUT4 content in the heart was not different among all other groups.

Plasma Insulin and Metabolite Levels in Control and MLC-GLUT4 Mice

Plasma glucose, lactate, FFA, and insulin levels were measured in saline-treated lean and GTG-treated obese control and MLC-GLUT4 mice following a 6-hour fast (Table 1). Lean control and MLC-GLUT4 mice exhibited similar glucose and insulin levels. Glucose and insulin levels did not differ in the GTG-treated obese MLC-GLUT4 group and the obese control group. Both insulin and glucose were increased in mice treated with GTG compared with saline-injected mice regardless of genotype. Insulin levels were 6-fold higher in GTG-treated obese MLC-GLUT4 and control mice versus saline-treated lean MLC-GLUT4 and control groups. No significant differences were detected in plasma lactate or FFA levels among all 4 groups of mice tested.

Table 1. Serum Metabolite Levels in 28-Week-Old Lean and Obese Control and MLC-GLUT4 Mice Following a 6-Hour Fast

Group	Insulin (ng/mL)	Glucose (mg/dL)	Lactate (mg/dL)	FFA (μEq/L)
Saline-lean MLC-GLUT4 (n = 5)	1.3 ± 0.2	168.0 ± 7.9	30.3 ± 5.4	982 ± 95
GTG-obese MLC-GLUT4 (n = 4)	8.3 ± 1.2†	217.9 ± 16.4*	29.2 ± 3.4	863 ± 65
Saline-lean control (n = 5)	1.2 ± 0.2	175.1 ± 8.8	25.4 ± 5.0	924 ± 45
GTG-obese control (n = 5)	8.3 ± 2.1†	219.8 ± 15.5*	29.2 ± 3.3	925 ± 69

NOTE. Data are the mean ± SE.

*$P < .02$, lean *v* GTG-treated obese.

†$P < .01$, lean *v* GTG-treated obese.

Insulin Tolerance Test

Whole-body insulin action was assessed by an insulin tolerance test (Fig 3). Following insulin injection, both saline-treated lean control and MLC-GLUT4 mice cleared glucose efficiently. Blood glucose levels in lean control and MLC-GLUT4 mice at 60 minutes following insulin injection were 38% and 63%, respectively, of the initial blood glucose values. Similarly, GTG-treated obese MLC-GLUT4 mice were able to clear blood glucose efficiently in response to insulin treatment. In GTG-treated obese MLC-GLUT4 mice, blood glucose decreased from 190 mg/dL to 113 mg/dL within 1 hour following insulin injection, representing a 40% decrease. In contrast, GTG-treated obese control mice were unable to clear blood glucose in response to insulin treatment. There was no difference in blood glucose levels in obese control mice before and 1 hour after insulin injection.

DISCUSSION

The consequences of GTG administration on weight gain, insulin action, and GLUT4 expression were studied in MLC-GLUT4 transgenic and control mice in the present investigation. A single injection of GTG resulted in accelerated weight gain in both control and MLC-GLUT4 mice. In GTG-treated obese control mice, administration of insulin did not result in a significant decrease of blood glucose, demonstrating an insulin-resistant state accompanied by obesity. In contrast, despite the development of obesity nearly equal in magnitude to the controls, GTG-treated obese MLC-GLUT4 mice were able to clear blood glucose efficiently following insulin injection. The ability of GTG-treated obese MLC-GLUT4 mice to decrease blood glucose under insulin stimulation was concomitant with a sustained increase in skeletal muscle GLUT4 content. These studies indicate that overexpression of GLUT4 in skeletal muscle can ameliorate the development of impaired insulin action associated with obesity.

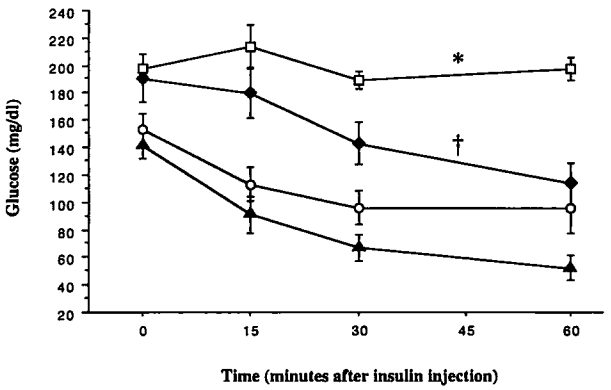

Fig 3. Insulin tolerance test. Porcine insulin (1.8 U/kg body weight) was injected intraperitoneally in conscious 28-week-old saline-treated lean control (○, n = 5), lean MLC-GLUT4 (▲, n = 5), GTG-treated obese control (□, n = 5), and obese MLC-GLUT4 (◆, n = 4) mice. Blood was withdrawn from the retro-orbital sinus at 0, 15, 30, and 60 minutes after injection, and the blood glucose level was monitored by a glucometer. Glucose clearance curves are significantly different between GTG-treated obese controls and all other groups (*$P < .02$) and between GTG-treated obese MLC-GLUT4 mice and all other groups (†$P < .02$) as determined by ANOVA for repeated measures using Fisher's PLSD post hoc analysis.

Alterations in skeletal muscle GLUT4 expression and/or function have been implicated in insulin-resistant states.[27-30] Although GTG-induced obesity results in impaired skeletal muscle glucose uptake,[6,31] previous studies have shown either no change[24] or only a modest decrease[32] in skeletal muscle GLUT4 content following GTG-induced obesity. No alteration was observed in skeletal muscle GLUT4 content between saline- and GTG-treated control mice in the present study. Together, these results suggest that defects in insulin-mediated GLUT4 translocation are responsible for impaired muscle glucose uptake. However, the restoration of normal glucose uptake may be achieved with GLUT4 overexpression.

Decreased GLUT4 expression in WAT has been shown to follow the onset of insulin resistance in obese and non-obese animal models of type 2 diabetes mellitus.[28,33-35] It has been demonstrated that GTG-induced obesity in mice is associated with significant downregulation of GLUT4 expression.[32] In accordance with these prior findings, we observed a severe decrease in WAT GLUT4 levels of GTG-treated obese control mice. It is noteworthy that this severe downregulation of GLUT4 expression was not found in WAT of GTG-treated obese but insulin-sensitive MLC-GLUT4 mice. This result suggests that decreased WAT GLUT4 expression, in part, is a consequence of whole-body insulin resistance. Previously, we observed in male mice heterozygous for GLUT4 disruption that the decrease in WAT GLUT4 content preceded the decrease in skeletal muscle GLUT4 content.[36] These prior findings and results of the present study are consistent with the notion that downregulation of WAT GLUT4 can significantly contribute to the onset and/or maintenance of impaired peripheral insulin action. The development of skeletal muscle insulin resistance is intimately connected to a sharp decrease in WAT GLUT4 content, indicating a dynamic interplay between skeletal muscle and WAT in the determination of whole-body insulin action. In addition, our group and others have demonstrated recently that WAT GLUT4 levels may affect WAT mass,[26,37,38] which in turn can influence whole-body lipid metabolism, insulin action, and glucose disposal.

Peripheral insulin resistance is associated with human obesity[22] and animal models of obesity.[23] In humans, an increased percentage of body fat is correlated with a decreased rate of glucose disposal.[39] In animals, decreased skeletal muscle glucose uptake has been demonstrated in mice with induced or genetic obesity.[6,40] The relationship between obesity and impaired insulin action has not been clearly defined. In GTG-treated MLC-GLUT4 mice, obesity is uncoupled from impaired insulin action, since they were nearly as obese as control mice treated with GTG yet were able to maintain insulin action/tolerance. This result suggests that the degree of obesity is only one of many factors controlling insulin sensitivity, and a selective increase in skeletal muscle GLUT4 content can overcome the negative influence of obesity on insulin action.

Insulin resistance and subsequent hyperinsulinemia has been proposed as a contributing factor in obesity.[41] It has also been proposed that insulin resistance is an adaptive response elicited during the development of obesity to prevent further weight

gain.[22,42] If this hypothesis is correct, it is reasonable to further hypothesize that an animal with high insulin sensitivity would be predisposed to gain more weight than an animal with low insulin sensitivity under obesity-promoting conditions. Previously, we showed that whole-body insulin-stimulated glucose utilization is increased in MLC-GLUT4 transgenic mice.[14] Despite the increased insulin action, MLC-GLUT4 mice did not gain more weight than control mice following GTG treatment. This finding suggests that rodents with enhanced insulin action are not necessarily predisposed to develop more severe obesity. However, it does not validate or disprove the hypothesis that insulin resistance is an adaptive response to prevent further weight gain.

Although normal insulin action was preserved in obese MLC-GLUT4 mice, glucose and insulin remained elevated compared with the levels in lean MLC-GLUT4 and control mice. This finding indicates that glucose clearance by skeletal muscle is only one factor responsible for the maintenance of glucose homeostasis. It further suggests that other factors contributing to abnormal glucose homeostasis in GTG-induced obesity cannot be corrected by expression of the MLC-GLUT4 transgene. This conclusion is supported by recent studies of transgenic mice overexpressing the human GLUT4 gene in the *db/db* background[21] and GLUT4-overexpressing transgenic mice treated with streptozotocin.[15] The *db* gene encodes a truncated leptin receptor, which is expressed at high levels in the hypothalamus.[43] GLUT4-overexpressing *db/db* mice exhibited improved glycemic control based on glucose tolerance tests. However, they remained hyperglycemic (approximately 350 mg/dL), although this represented a significant improvement in blood glucose as compared with *db/db* mice not overexpressing GLUT4. When streptozotocin was administered to transgenic mice overexpressing GLUT4 selectively in skeletal muscle, insulin action was not as impaired as in similarly treated control mice.[15] However, blood glucose in these streptozotocin-treated GLUT4-overexpressing mice remained at diabetic levels. These results indicate that the protective effect of GLUT4 on the maintenance of whole-body glucose homeostasis is limited. Thus, gene-therapy strategies for the treatment of type 2 diabetes should address the defects in skeletal muscle glucose utilization, as well as other defects such as impaired suppression of hepatic glucose output. In addition, the inability of GLUT4 overexpression to normalize basal blood glucose levels in *db/db,* streptozotocin-treated, and GTG-treated obese mice suggests that the abnormal regulation of another glucose transporter may be involved. Recent studies have shown that GLUT1 overexpression in the skeletal muscle of transgenic mice leads to increased basal glucose uptake, lending support to the hypothesis that GLUT1 may play a significant role in the regulation of basal glucose transport.[44-46]

A currently unresolved issue is the contribution of insulin resistance to the development and maintenance of hyperinsulinemia in animal models of obesity. Hyperinsulinemia has been shown to precede the dynamic phase of weight gain and insulin resistance in animal models of hypothalamic and genetic obesity.[23] However, the onset of insulin resistance may play a significant role in maintaining hyperinsulinemia and further exacerbating its severity. Compared with GTG-treated obese control mice, similarly treated obese MLC-GLUT4 mice exhibited markedly improved glucose clearance in response to insulin. Yet these obese MLC-GLUT4 mice exhibited the same degree of hyperinsulinemia as the obese control group. This finding suggests that insulin resistance does not likely play a predominant role in either the maintenance or deterioration of hyperinsulinemia. These results are consistent with impaired pancreatic β-cell function leading to hyperinsulinemia as a primary lesion in the development of insulin resistance and obesity-related type 2 diabetes.[47] The development of hyperinsulinemia may be due to a disruption of the autonomic nervous control of insulin secretion.[23,48,49] In addition, normal glucose-sensing and other β-cell functions may be altered by the increased islet triglyceride and FFA levels associated with obesity.[50,51]

In conclusion, we have induced obesity in both control and MLC-GLUT4 mice by GTG treatment. Whereas control mice developed insulin resistance following the onset of obesity, MLC-GLUT4 mice exhibited normal insulin action despite being nearly as obese. Normalization of insulin action was accompanied by skeletal muscle GLUT4 overexpression. The present study suggests that the correction of GLUT4 expression and/or function in skeletal muscle should be considered a viable strategy in the treatment of obesity-associated insulin resistance. However, since muscle GLUT4 overexpression and restoration of insulin action failed to correct the elevated glucose levels and hyperinsulinemia, other molecular and cellular targets, including WAT GLUT4 and factors affecting β-cell function, must be considered as well.

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