Glucokinase Gene Transfer to Skeletal Muscle of Diabetic Zucker Fatty Rats Improves Insulin-Sensitive Glucose Uptake

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Skeletal muscle has a prime role in glucose homeostasis. We have previously demonstrated that adenovirus-mediated glucokinase (GK) gene transfer to skeletal muscle of Wistar rats enhances muscle glucose uptake and whole body glucose disposal under conditions of hyperglycemia and hyperinsulinemia. In this study, we have tested whether GK gene transfer to the muscle of the Zucker Diabetic Fatty rat (ZDF), a genetic model of obesity and type 2 diabetes, could improve glycemic control and prevent the onset of hyperglycemia in obese males. We show that GK delivery results in a doubling of total gastrocnemius muscle glucose phosphorylating activity 9 weeks after gene transfer. GK-treated rats exhibited slightly reduced weight and normal insulin-sensitive glucose uptake, as assessed during an insulin tolerance test, whereas age-matched rats treated with a control virus were clearly insulin resistant. The improved glucose uptake in GK-expressing rats was associated with higher gastrocnemius lactate content, whereas glycogen and triacylglyceride (TAG) levels were unmodified. Remarkably, GK-treated rats showed increased expression of both hexokinase II (HKII) and GLUT4, in accordance with a glucose-dependent regulation of these proteins. Thus, our data show that delivery of GK, despite improving insulin-sensitive glucose disposal in muscle, is not sufficient to prevent or delay the appearance of elevated glucose and insulin levels associated with severe obesity in male ZDF animals.

A MAJOR INITIAL DEFECT in type 2 diabetes is the impaired response of tissues (muscle, liver, and fat) to insulin action. Zucker diabetic fatty rats (ZDF fa/fa) are a genetic model of obesity and type 2 diabetes, resulting from a colony of Zucker rats, which bear a mutation in the leptin receptor.[1] Both homozygous ZDF males and females are insulin resistant, but only males develop hyperglycemia, beginning at 7 to 8 weeks of age. The relative contribution of peripheral tissues and liver to development of insulin resistance in the ZDF model has not been defined. Nevertheless, it is widely accepted that skeletal muscle has a prime role in the instauration of the diabetic phenotype. Consistently, muscle glucose uptake was found to be markedly impaired in soleus, gastrocnemius, and diaphragm muscles of ZDF rats.[2] However, this impairment was not explained by reduced expression of the insulin-regulated GLUT4 glucose transporter in muscle, as ZDF and lean control rats (ZL) contained similar amounts of GLUT-4 protein in white gastrocnemius, whereas a slight decrease in GLUT4 was described in red soleus muscle.[3]

Insulin-stimulated glucose uptake is also limited, in certain metabolic conditions, by the hexokinase II (HKII)-catalyzed phosphorylation of the sugar,[4-6] due to the autoinhibition of HKII by its own product, glucose 6-phosphate.[7,8] Our previous work has provided new evidence in support of this concept, as expression of glucokinase (GK), a glucose-6-phosphate-insensitive HK isoform, in muscle led to an enhancement in glucose disposal during a glucose tolerance test performed in fed Wistar rats.[9] In addition to its lack of sensitivity to glucose-6-phosphate as an allosteric inhibitor, GK also differs from other HK isoforms, such as HKII, in that it has a higher Km for glucose.[10] These kinetic features of GK appear to explain how the enzyme can enhance muscle glucose disposal under conditions in which glucose transport is maximally stimulated, eg, hyperinsulinemia and hyperglycemia. Based on these findings, we have now investigated whether GK overexpression in muscle of male ZDF rats can improve obesity-associated insulin resistance and glucose disposal. This was accomplished by adenovirus-mediated delivery of the GK cDNA to muscle of ZDF rats. We show that this maneuver improves insulin-sensitive glucose uptake, but that these effects are not sufficient to restore normal glucose or insulin levels. This suggests that muscle insulin resistance is not a major cause of hyperglycemia and hyperinsulinemia characteristically found in the obese ZDF males.

MATERIALS AND METHODS

Recombinant Adenovirus Preparation and Administration to Animals

Recombinant adenoviruses containing the cDNA encoding rat liver glucokinase (AdGK)[11] or the *Escherichia coli* β-galactosidase nls-LacZ gene (AdLacZ)[12] have been described previously. Viruses were amplified in 293 cells and concentrated to 10^{11} to 10^{12} pfu/mL by centrifugation through a CsCl gradient. Viruses were aliquoted and stored at −70°C in phosphate-buffered saline (PBS). Viruses were titrated by a serial dilution plaque-assay in noble agar overlayed 293 cells. Approximately 10^{10} pfu of AdGK or AdlacZ were delivered to newborn (2 to 3 days) ZDF rats by 3 intramuscular (IM) injections, at the same time and separate sites, in gastrocnemius of both hind legs. All experiments were performed in at least 3 separate batches of littermates, and only males were included in the study.

Animals were maintained at 23°C with a 12-hour light-dark cycle.

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They were weaned at 22 days and thereafter provided with a standard chow diet and water ad libitum. When stated, animals were fasted by 24-hour deprivation of food. Animals were killed after anesthesia with pentobarbital. Gastrocnemius muscles were excised and immediately frozen in liquid N_2 followed by freeze-drying in an Alpha 1-4/RVC drier (B. Braun, Inc, New York, NY) and stored at -20°C. Animals treated with AdGK or AdLacZ were analyzed for transgene expression in muscle.

Western Blot Analysis and Glucose Phosphorylating Activity (GK, HKI, and HKII)

Freeze-dried muscles were cut very finely. Samples of 10 mg were homogenized in 50 vol of ice-cold buffer 50 mmol/L Tris-HCl buffer (pH 7.4) with 1 mmol/L EDTA, 100 mmol/L potassium chloride (KCl), 300 mmol/L sucrose, 10 mmol/L β-mercaptoethanol, 1 mmol/L leupeptin, 1 mmol/L benzamidin, and centrifuged at 10,000×g at 4°C for 15 minutes. The resulting supernatants were used for the determination of enzyme activities and Western blot analysis. Protein concentration was measured with Bio-Rad (Barcelona, Spain) protein assay reagent. For Western blotting, samples of 20 μg of protein were prepared by standard procedures and immunoblotting performed using a polyclonal antibody as described.[11,13] Crude membranes for GLUT4 analysis were prepared and immunoblot analysis performed as previously described.[14] Polyclonal anti-GLUT4 antibody was kindly provided by Dr A. Zorzano (Universitat de Barcelona, Spain) and anti-HKII antibody was from Clontech Biotech (Barcelona, Spain).

Total glucose phosphorylating activity was determined at 100 mmol/L glucose as described[15] in a Cobas Fara II autoanalyzer (Barcelona, Spain). Every single HK was estimated as follows: glucokinase activity was obtained as the resulting activity of substracting the glucose phosphorylating capacity at 10 mmol/L glucose with regard to the capacity at 100 mmol/L. HKI activity was determined by heating the muscle extract at 45°C during 1 hour before the assay at 10 mmol/L glucose. HKII was obtained by substracting the activity assayed at 10 mmol/L glucose from the activity after heating the extract at 45°C during 1 hour.[16]

Measurement of Metabolite and Insulin

Serum insulin levels were assayed by enzyme-linked immunosorbent assay (ELISA) (Crystal Chem Inc, Chicago, IL). Serum triacylglycerides (TAGs) and free fatty acids (FFAs) were measured using kits from Sigma Chemical, St Louis, MO. Blood glucose was monitored with the aid of a Reflotron photometer (Lifescan, Johnson & Johnson, New York, NY).

For metabolite measurements in muscle, samples of 20 mg of dry gastrocnemius were finely powdered and homogenized in 20 vol of potassium hydroxide (KOH) 30%, HClO4 10%, or buffer for lipid extraction (100 mmol/L KCl, 20 mmol/L KF, 0.5 mmol/L EDTA, 0.05% Lubrol, pH 7.9) for the measurement of glycogen, lactate, and TAG, respectively. Samples were centrifuged at 10,000×g at 4°C during 15 minutes and metabolite content was assayed in the supernatant. Glycogen, lactate, and TAGs were measured enzymatically in a Cobas Fara II autoanalyzer by adapting kits from Sigma Chemicals Co.

Insulin Tolerance Test

To test insulin tolerance, 18-hour–fasted animals were anesthetized with pentobarbital, and an insulin bolus (1.2 U/kg/body weight) was administered by intraperitoneal (IP) injection. Animals were subsequently bled via the tail vein. Blood glucose concentration was measured as described previously. Insulin was prepared as follows: porcine insulin was prepared at a final concentration of 0.5 U/mL in a solution that contained 0.2% bovine serum albumin (BSA) and 10 mmol/L HCl.

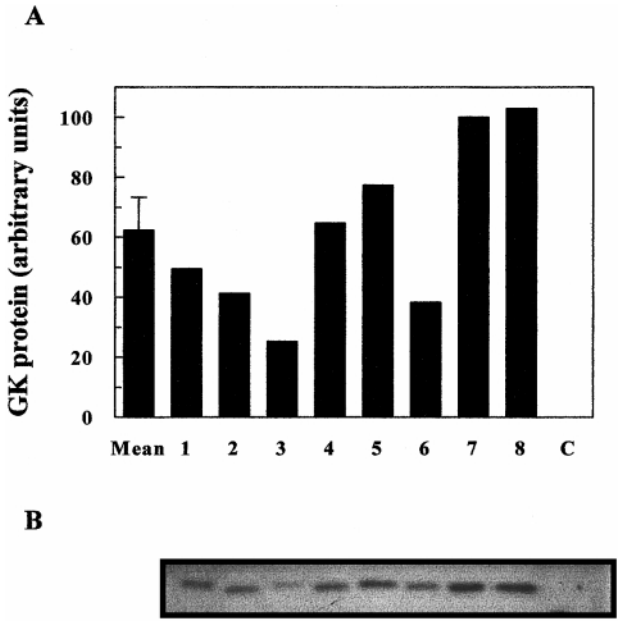

Fig 1. Expression of GK in gastrocnemius muscle. Immunoblot analysis of samples (20 μg of protein) of gastrocnemius muscle from AdGK or AdLacZ-treated rats. The relative expression of GK protein was quantified by densitometric analysis of the immunoblots (A). Immunoblots from 8 GK-treated muscles and a representative control (C) from LacZ-treated muscle are shown (B). The individual value for every muscle and the mean value ± SEM of the GK-treated muscles (n = 8) are shown.

Statistical Analysis

Data are expressed as means ± SE. The statistical significance was determined by an unpaired Student's t test.

RESULTS

HK Expression in Skeletal Muscle

The AdGK adenovirus containing the rat liver GK cDNA was injected IM into the hindleg of newborn rats as previously described.[9] In the present study, both leg gastrocnemius were injected, with each leg receiving 3 independent injections, with a total dose of 10^{10} pfu/animal of AdGK or AdLacZ virus. With this protocol, the expression of the transgene, as revealed by histochemical detection of the reporter protein β-galactosidase, was maintained for 10 weeks after delivery in approximately 60% of fibers in the gastrocnemius muscle of the injected legs, as well as other contiguos muscles in the leg posterior lodge with varying intensities (data not shown). Analysis of GK gene delivery and metabolic impact was restricted to the injected gastrocnemius muscle. Nine weeks after injection of AdGK, GK protein content was measured in gastrocnemius muscle by immunoblot analysis. The enzyme was present in samples from all AdGK-injected rats and absent from all controls. GK protein content varied from muscle to muscle over a 5-fold range (Fig 1). Glucose phosphorylating activity was assayed at a saturating glucose concentration (100 mmol/L) in muscle extracts from the AdGK-treated and AdLacZ-treated rats. In GK-expressing animals, total mean HK activity was doubled when

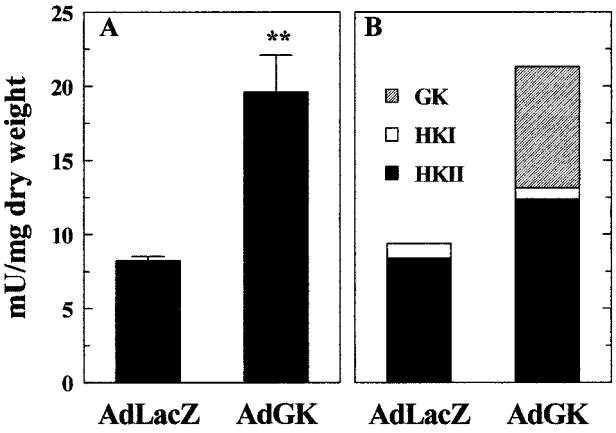

Fig 2. Glucose phosphorylating activity. (A) Total glucose phosphorylating activity measured at a glucose saturating concentration (100 mmol/L),in muscle extracts of gastrocnemius muscles from AdGKL- (n = 7) and AdLacZ-treated rats (n = 4). Values are means ± SEM. (B) HK activities (I, II, and GK) were determined as described in Materials and Methods. Data represent the contribution of every HK activity to the total phosphorylating activity. The significance of the difference is **$P < .001$ as compared with AdLacZ.

compared with the activity in control muscle extracts (Fig 2A). When the contribution of each HK isoform to total HK activity was estimated (Fig 2B), it was found that GK activity was 8.01 ± 1.59 mU/mg in the GK-expressing animals. HKI was unmodified in all muscles with a mean value of 0.81 ± 0.07 mU/mg dry weight. Interestingly, HKII activity, which was estimated at 9.37 ± 0.99 mU/mg dry weight, was increased by 140% in muscle samples from GK-expressing animals (ZL-GKL and ZDF-GKL) relative to controls. This effect was associated with a marked increase of about 3-fold in the content of HKII protein as assessed by Western blot (Fig 3), suggesting that expression of GK in muscle increased HKII expression in this animal model. Similarly, total GLUT4 protein was highly increased (more than 4-fold) in GK-expressing muscles compared with controls. In contrast, GLUT1 protein levels were unchanged in the 2 groups (data not shown). Thus, overexpression of GK in muscle increased the expression of GLUT4 in concert with its effects on HKII.

Body Weight and Serum Metabolites

Body weight, glucose, TAG, and FFA were monitored in ad libitum-fed rats from the 6th week of age (Fig 4). A statistically significant decrease in weight (Fig 4A) was observed in the GK-expressing group. In contrast, hyperglycemia (Fig 4B), hypertriglyceridemia (Fig4C), and increases in FFA (Fig 4D) developed in all animals between the 7th and 9th weeks of age without statistically significant differences between animals that received the AdGK or control virus. Moreover, insulin levels in fed GK-treated rats (8.2 ± 0.3 ng/mL) of 9 weeks of age were indistinguishable from those in LacZ-treated animals (7.8 ± 1.5 ng/mL). Neither was fasting glucose different in lacZ- (91 ± 5 mg/dlL and GK-treated (97 ± 6 mg/dL) animals at the 9th week of age.

Insulin Tolerance

To evaluate insulin-sensitive glucose uptake, an IP insulin tolerance test (ITT) was performed at the 9th week of age in fasted rats (Fig 5). As expected, ZDF rats with AdLacZ virus were clearly insulin resistant, as glucose levels declined by only 30%, 90 minutes after the insulin bolus. GK-treated animals exhibited an improvement in insulin response, with a 50% decrease in blood glucose concentration over the same time interval. Importantly, the insulin response of the GK-expressing ZDF rats was equivalent to that observed in the noninsulin-resistant lean heterozygous males (data not shown).

Impact on Muscle Metabolism

Muscle metabolites, such as lactate, glycogen, and TAG, were measured in gastrocnemius samples at the end of the IPITT (Table 1). Glycogen content and TAG levels were similar in AdGK- and AdLacZ-treated animals. In contrast, IM lactate concentration was approximately twice as high in AdGK-treated rats as in AdLacZ-injected controls. These data indicate that the improvement in insulin-sensitive glucose uptake observed in the GK-treated group is due to an increase in glycolytic flux, resulting in enhanced muscle glucose disposal.

DISCUSSION

We have previously shown that GK delivery to the muscle of normal, nondiabetic Wistar rats increases muscle glucose uptake under hyperglycemic/hyperinsulinemic conditions, lead-

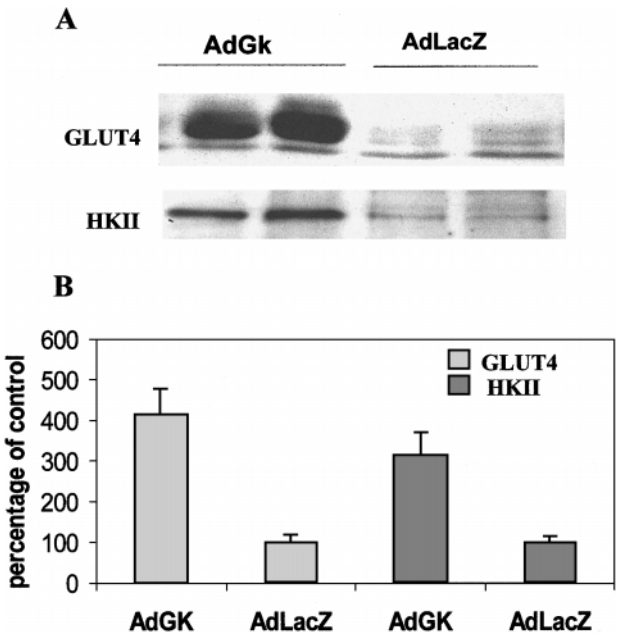

Fig 3. Expression of HKII and GLUT4 in gastrocnemius muscle. Immunoblots of total protein extracts from gastrocnemius muscles from LacZ-treated (AdLacZ, n = 6) and GK-treated rats (AdGK, n = 6) resected at the end of the ITT. Representative muscles are shown (A). The relative expression of GLUT4 and HKII proteins were quantitated by densitometric analysis of the immunoblots (B). Data are means ± SEM and are expressed in percentage terms of the values for AdLacZ-treated animals.

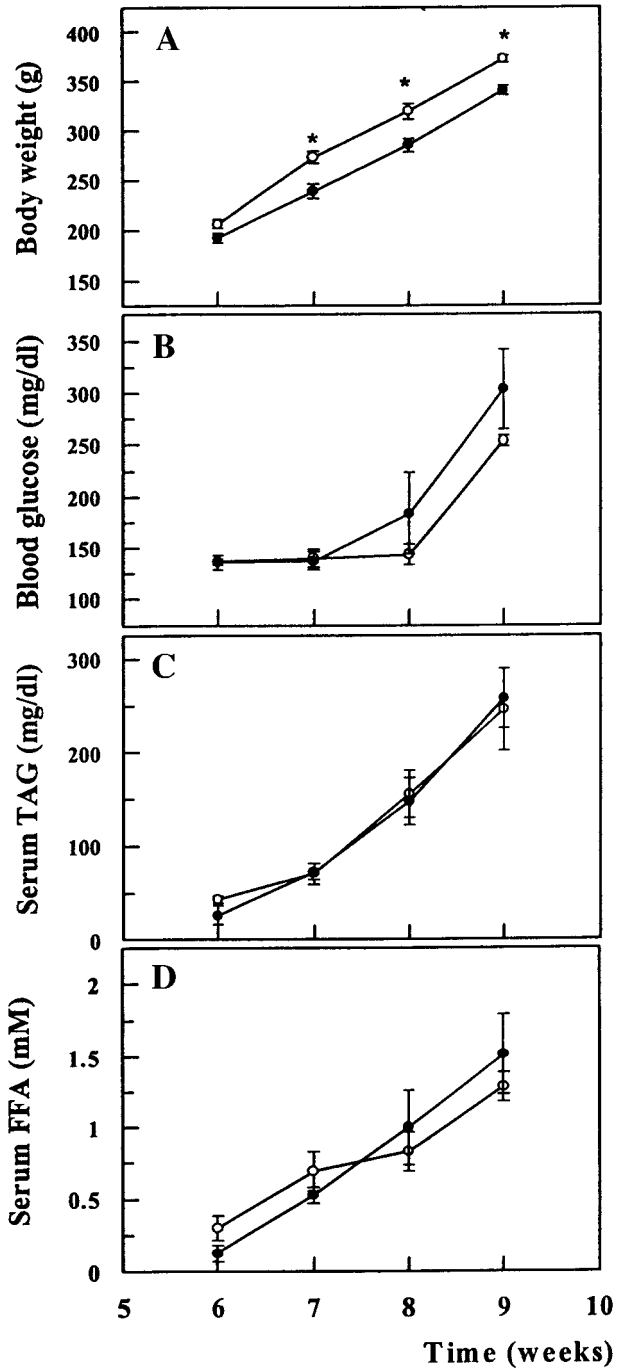

Fig 4. Body weight and serum metabolites. Body weight (A), blood glucose (B), serum TAG (C), and FFA (D) were monitored in ad libitum-fed LacZ-treated (○, n = 5) and GK-treated rats (●, n = 7) from the 6th week of age. Values are means ± SEM, and the significance of the difference is *$P < .05$ as compared with AdLacZ-treated rats.

ing to an enhancement in whole body glucose disposal.[9] In the current study, we have investigated whether GK gene expression in muscle of ZDF males is sufficient to improve glycemic control and prevent the onset of hyperglycemia linked to obesity.

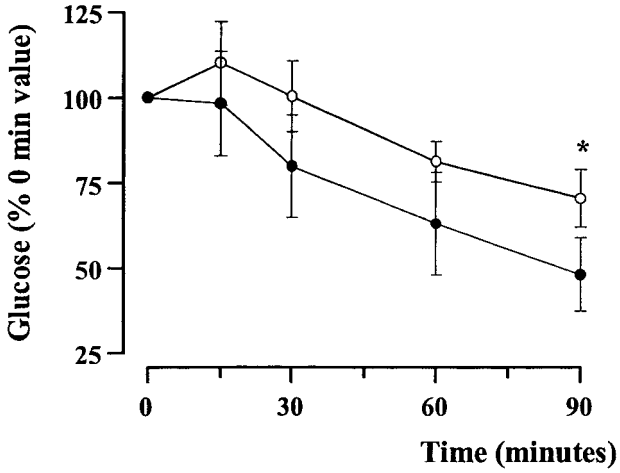

Fig 5. ITT. An IP insulin bolus (1.2 U insulin/kg/body weight) was administered to anestethized rats after an overnight fast. Glucose was measured in blood samples from the tail vein as described in Materials and Methods, and values are presented as the percentage of initial glucose value. Values are means ± SEM from AdLacZ-treated (○, n = 4) and AdGK-treated rats (●, n = 4). The significance of the differences is *$P < .05$ as compared with AdLacZ-treated rats.

IM injection of adenovirus to newborn ZDF rats allowed substantial expression of GK in muscle (around 8 mU/mg dry weight in gastrocnemius) of 2-month-old rats, the age at which ZDF males develop frank hyperglycemia. Insulin-sensitive glucose uptake, as assessed by an IPITT, was clearly improved in GK-treated animals. This enhancement in insulin-stimulated glucose disposal was associated with an increase in the IM lactate content, as measured in gastrocnemius, at the end of the IPITT, suggesting that the engineered muscles had a higher glycolytic rate. In contrast, no changes in glycogen content were found in the GK-expressing gastrocnemius muscles compared with controls, consistent with our earlier findings in normal Wistar rats. The in vivo data contrast with the increase in glycogen that occurs when GK is delivered to muscle cells in culture.[17] This apparent discrepancy may be due to the lower level of GK activity achieved by adenovirus-mediated gene delivery in vivo (activity increased 2-fold) as compared with that accomplished in vitro (activity increased 13-fold). An interesting finding of the current study is that the insulin-sensitive proteins involved in muscle glucose uptake, GLUT4 and HKII, were upregulated in response to GK expression, whereas GLUT1 and HKI were unaltered. This observation

Table 1. IM Metabolites Content

	AdLacZ	AdGK
Glycogen (mg glucose/mg weight)	21.4 ± 1.2	17.2 ± 1.6
TAG (mg glycerol/mg weight)	636 ± 300	721 ± 260
Lactate (μg/mg weight)	0.92 ± 0.15	2.12 ± 0.35*

NOTE. Glycogen, TAGs, and lactate concentration were determined in gastrocnemius muscles 90 minutes after insulin injection. Values are means ± SEM from AdLacZ-treated (n = 7) and AdGK-treated rats (n = 7).

*The significance of the difference is $P < .05$.

strengthens the notion that one of the control mechanisms of GLUT4 and HKII expression is a glucose-derived metabolic signal.[8,18] In this same line of evidence, we have previously shown that GK expression (unpublished results) or glycogen phosphorylase overexpression[14] in cultured human muscle cells caused the upregulation of GLUT4 mRNA and protein levels in association with enhanced glycolytic flux. Furthermore, GLUT4 has been shown to increase after activation of adenosine monophosphate (AMP) protein kinase,[19] which is involved in the stimulation of gene expression by glucose.[20] In our previous study, neither GLUT4 or HKII upregulation was observed after in vivo GK delivery to muscle of nondiabetic Wistar rats.[9] Because the GK activity levels attained in the Wistar and ZDF rats by adenovirus-mediated delivery were similar, it is suggested that other unidentified factors related to the genetic background of the ZDF rat may be involved. Nevertheless, we may speculate that elevated levels of insulin and glucose in the ZDF rats may play a permissive role for the glucose-derived metabolic signal. On the other hand, it is also possible that lipid overaccumulation in muscle of ZDF rats is impairing normal glucose-mediated regulation of GLUT-4 and HKII expression, and that this pathway is restored upon overexpression of GK in muscle. Clearly, further work will be needed to clarify the operative mechanisms.

Despite the improved whole body insulin-sensitive glucose uptake, expression of GK failed to alter any of the serum metabolites measured in ZDF animals in the basal state, relative to ZDF rats treated with the AdLacZ control virus. Thus, indicating that in this model, the improvement in skeletal muscle glucose uptake is not sufficient to reverse the diabetic phenotype. Nevertheless, a reduction in weight was observed in GK-treated animals, consistent with a higher proportion of glucose consumption occuring in muscle versus adipose tissue. Presumably, the adipose tissue makes a major contribution to insulin resistance in the morbid obese ZDF rats.[21] Similar conclusions were raised in transgenic mice overexpressing GLUT4 selectively in skeletal muscle and subjected to gold-thioglucose injection, which caused obesity and hyperinsulinemia.[22] Genetic overexpression of GLUT4, in this model, ameliorated obesity-associated insulin resistance, but could not restore normal glucose and insulin levels. In contrast, in studies in the db/db mouse, which also bears a mutation in leptin receptor, a marked improvement in hyperglycemia was found when they were crossed to hGLUT4 minigene transgenic mice, which ovexpressed GLUT4 in muscle, as well as adipose tissue.[23] These animals showed better glycemic control, although different from what occurred in our model, they also exhibited an increase in body weight and adiposity. Similarly, treatment of ZDF rats with the peroxisome proliferator-activated receptor (PPAR)-γ agonist, GW1929, lowered blood glucose levels and improved insulin sensitivity, while normalizing TG and nonesterified fatty acids (NEFA) levels.[24] Again, this effect was mainly mediated by changes in adipose tissue and was correlated to a marked gain in body weight. Here, we show that the improvement in muscle insulin-sensitive glucose disposal achieved by GK expression in the morbid obese ZDF males, although resulting in repartioning of nutrients towards muscle and reducing weight, is not sufficient to correct hyperglycemia and hyperinsulinemia.

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