Original Article

Reversal of Type 1 Diabetes by Engineering a Glucose Sensor in Skeletal Muscle

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Type 1 diabetic patients develop severe secondary complications because insulin treatment does not guarantee normoglycemia. Thus, efficient regulation of glucose homeostasis is a major challenge in diabetes therapy. Skeletal muscle is the most important tissue for glucose disposal after a meal. However, the lack of insulin during diabetes impairs glucose uptake. To increase glucose removal from blood, skeletal muscle of transgenic mice was engineered both to produce basal levels of insulin and to express the liver enzyme glucokinase. After streptozotozin (STZ) administration of double-transgenic mice, a synergic action in skeletal muscle between the insulin produced and the increased glucose phosphorylation by glucokinase was established, preventing hyperglycemia and metabolic alterations. These findings suggested that insulin and glucokinase might be expressed in skeletal muscle, using adeno-associated viral 1 (AAV1) vectors as a new gene therapy approach for diabetes. AAV1-Ins+GK–treated diabetic mice restored and maintained normoglycemia in fed and fasted conditions for >4 months after STZ administration. Furthermore, these mice showed normalization of metabolic parameters, glucose tolerance, and food and fluid intake. Therefore, the joint action of basal insulin production and glucokinase activity may generate a "glucose sensor" in skeletal muscle that allows proper regulation of glycemia in diabetic animals and thus prevents secondary complications. *Diabetes* 55:1546–1553, 2006

Type 1 diabetes results from the autoimmune destruction of insulin-producing β-cells in pancreatic islets. Patients develop hyperglycemia and need insulin replacement therapy to survive (1). However, this therapy is imperfect because glycemia is not always appropriately regulated, and chronic hyperglycemia leads to microvascular, macrovascular, and neurological complications (2). Intensive insulin therapy can delay the onset and slow the progression of secondary complications, but patients receiving this therapy present a high risk of hypoglycemia (3). To maintain normoglycemia, pancreas and islet transplantation have also been used as alternatives for restoring endogenous insulin secretion (4). However, the limited availability of pancreatic tissue donors and the potential autoimmune reactions are severe restrictions. Thus, currently there is extensive research on endocrine pancreas regeneration and stem cell–derived β-cells (5,6). In addition, studies are also focused on the use of surrogate non–β-cells to deliver insulin (6,7). These approaches aim to lower blood glucose by delivering insulin under the control of glucose-responsive promoters, such as pyruvate kinase in the liver (8). However, the slow transcriptional control by glucose delays the insulin secretory response, which may lead to hyperglycemia immediately after meals and to hypoglycemia several hours later. To some extent, this can be circumvented by the use of cells that process and store insulin, such as gut K cells (9), or by inducing β-cell neogenesis in the liver by expression of the transcription factor *Neuro*D/*β2* (10). However, these strategies present other restrictions, such as feasibility, safety, and long-term efficacy.

To develop a more feasible approach, we have examined the ability of genetic manipulation of skeletal muscle to counteract diabetic hyperglycemia. Skeletal muscle is the most important site of glucose removal from blood, accounting for ~70% of glucose disposal after a meal. In addition, skeletal muscle is an excellent target tissue for gene therapy of type 1 diabetes because of its accessibility and its capacity to secrete proteins. Glucose utilization by skeletal muscle is controlled by insulin-stimulated glucose transport through GLUT4 (11) and its phosphorylation by hexokinase II (12). Hexokinase II has a low K_m for glucose and is inhibited by glucose-6-phosphate, which limits glucose uptake. During diabetes, because of the lack of insulin, GLUT4 translocation to the plasma membrane and hexokinase II mRNA levels and activity decrease (13,14). Expression of basal levels of insulin in skeletal muscle of transgenic mice increases glucose uptake (15) because insulin receptors are widely distributed in muscle fibers (16). When diabetic, these insulin-expressing transgenic mice are normoglycemic during fasting but remain hyperglycemic under fed conditions (16). To increase glucose phosphorylation, the hepatic glucose-phosphorylating enzyme glucokinase has also been expressed in skeletal muscle (17). In contrast to hexokinase II, glucokinase has a high K_m for glucose (~8 mmol/l), it is not inhibited by glucose 6-phosphate, and it shows kinetic cooperativity with glucose (12). These features allow glucose to be taken

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CMV, cyotmegalovirus; GFP, green fluorescent protein; STZ, streptozotozin.

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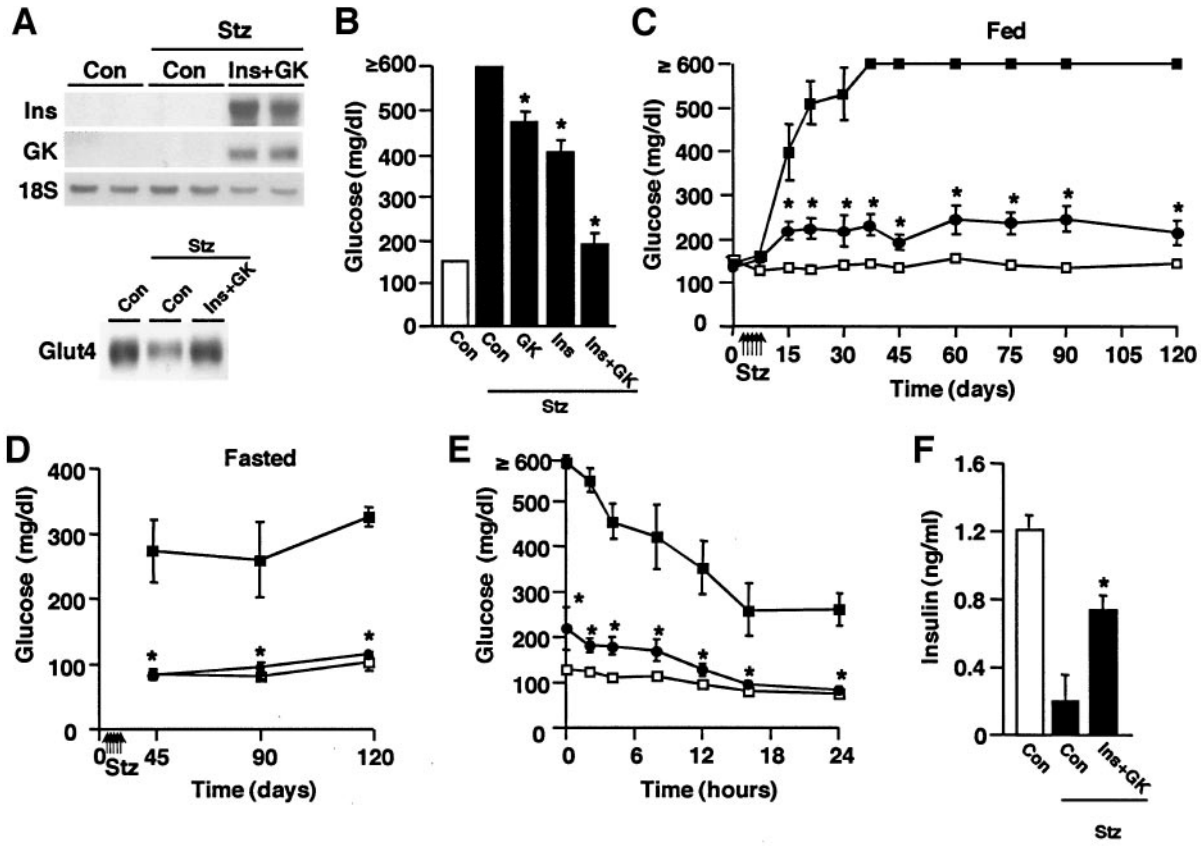

FIG. 1. Coexpression of insulin and glucokinase in skeletal muscle of STZ-administered transgenic mice counteracted diabetic hyperglycemia. *A*: Expression of insulin (Ins) and glucokinase (GK) in skeletal muscle. Total RNA was obtained from skeletal muscle, analyzed by Northern blot, and hybridized with insulin or glucokinase. A representative Northern blot is shown (*upper panel*). Western blot analysis of GLUT4 protein in skeletal muscle (*bottom panel*). *B*: Fed blood glucose levels in healthy control and in control, transgenic (insulin or glucokinase), and double-transgenic (Ins+GK) mice 6 weeks after STZ administration. At 4 months after STZ administration, fed (*C*) double-transgenic mice showed a mild increase in glycemia and normalization of fasted (*D*) glucose concentration. *E*: STZ-administered double-transgenic mice did not develop hypoglycemia after a 24-h fast. *F*: Serum insulin levels in healthy control (Con), STZ-administered control (STZ-Con), and double-transgenic (STZ-Ins+GK) mice were determined 4 months after STZ administration. All results are the means ± SE of 12 mice in each group. *P < 0.05 vs. STZ-control. □, healthy control mice; ■, STZ-administered control mice; ●, STZ-administered double-transgenic mice.

up only when it is at high concentrations, as previously reported in pancreatic β-cells (18). Expression of glucokinase in skeletal muscle increases glucose disposal and reduces diabetic hyperglycemia (17,19,20). However, expression of glucokinase alone cannot normalize glycemia in type 1 diabetes because of the lack of insulin-mediated glucose transport. Here, we examine whether the action of insulin and glucokinase is synergic in skeletal muscle. We hypothesized that because insulin produced by skeletal muscle increases glucose transport into muscle fibers, coexpression of glucokinase would increase glucose phosphorylation and utilization, leading to normoglycemia.

RESEARCH DESIGN AND METHODS

Heterozygous male double-transgenic mice (C57BL6/SJL) expressing both insulin and glucokinase in skeletal muscle were obtained by crossing transgenic mice expressing human proinsulin, containing genetically engineered furin endoprotease cleavage sites to produce mature insulin in skeletal muscle, with glucokinase-expressing transgenic mice (16,17). Skeletal muscle of the insulin-expressing transgenic mice produces ~80% of mature insulin and only ~20% of proinsulin (16). CD1 male mice were used for in vivo gene-transfer studies. Mice were fed ad libitum with a standard diet (Panlab, Barcelona, Spain) and maintained under a 12-h light/dark cycle (lights on at 8:00 A.M.). When stated, mice were fasted for 16 h. To induce insulin-dependent diabetes, mice aged 8 weeks were given, on 5 consecutive days, an intraperitoneal injection of streptozotocin (45 mg/kg body wt) dissolved in 0.1 mol/l citrate buffer (pH 4.5) immediately before administration. Diabetes was assessed by measuring blood glucose levels with a Glucometer Elite (Bayer, Leverkusen, Germany). Animal care and experimental procedures were approved by the ethics committee in animal and human experimentation of the Autonomous University of Barcelona.

Adeno-associated vectors. Adeno-associated viral 2/1 (AAV2/1) pseudotyped vectors were prepared by cotransfection in 293 cells of pAAV2(CMV/GFP), pAAV2(CMV/Ins), or pAAV2(CMV/GK); pLT-RCO2 encoding adenovirus helper functions (kindly provided by Dr. R. Mulligan, Harvard University); and pAAV1pITRCO2, which contains the AAV2 rep and AAV1 cap genes. Vector particles were purified on double cesium chloride gradients from cell lysates obtained 72 h after transfection, and titers were measured by quantitative dot-blot hybridization (21). The vector preparation used in this study had a titer of 2×10^{12} vector genomes/ml. Diabetic CD-1 mice were injected with 30 μl of PBS containing several doses of viral vectors into tibialis cranealis, gastrocnemius, and quadriceps muscles of both hindlimbs. All animal procedures were performed under appropriate biological containment.

RNA and protein analysis. Total RNA was obtained from skeletal muscle or liver samples and analyzed by Northern blot as described previously (16). Northern blots were hybridized to ^{32}P-labeled proinsulin, glucokinase, or PEPCK cDNA probes (16,17) labeled following the method of random oligopriming, as described by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). To determine GLUT4 expression, skeletal muscles were homogenized in lysis buffer. Western blot analysis was performed on protein from total membrane fraction, using a rabbit anti-mouse GLUT4 antibody (kindly provided by Dr. A. Zorzano, University of Barcelona).

Hormone and metabolite determinations. Total insulin levels in serum and in pancreas extracts were determined by radioimmunoassay (CIS Biointernational, Gif-Sur-Yvette, France). Mouse insulin levels where measured by enzyme-linked immunosorbent assay (Crystal Chemical, Chicago, IL). Serum triglycerides, free fatty acids, and β-hydroxybutyrate levels were measured as described previously (16). To determine the concentration of glycogen, skeletal muscle samples were clamped, frozen in situ, and kept at −80°C until analysis. The concentration of glycogen was measured in perchloric extracts

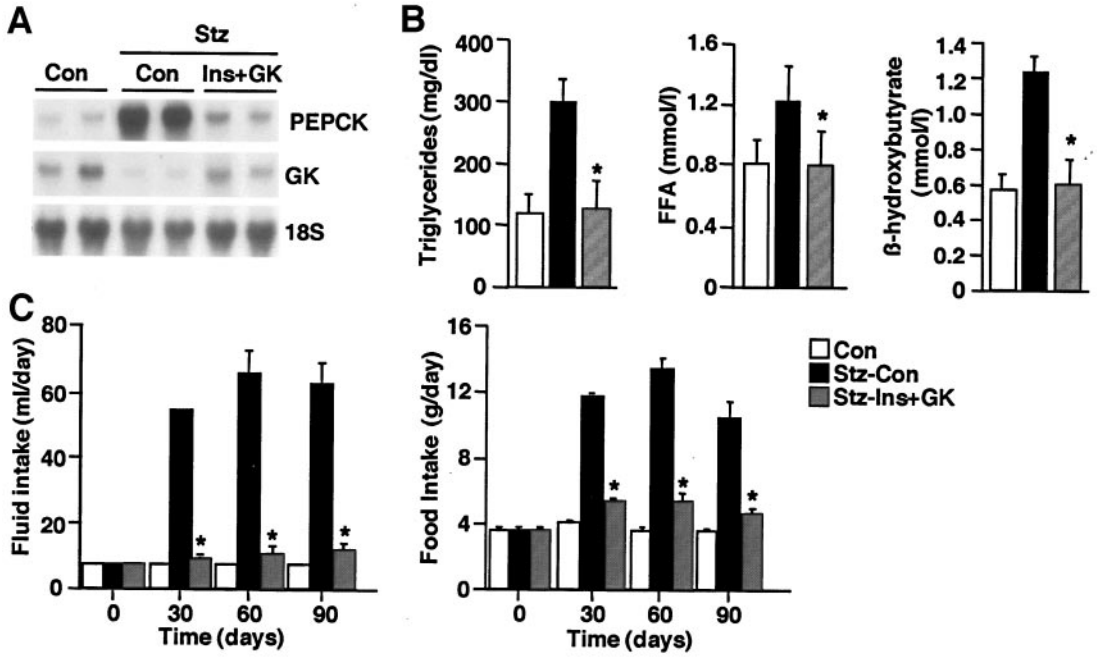

FIG. 2. Normalization of metabolic parameters in STZ-administered diabetic transgenic mice. *A*: Hepatic expression of PEPCK and glucokinase. A representative Northern blot is shown. *B*: Serum triglycerides, free fatty acids, and β-hydroxybutyrate were determined 4 months after STZ administration. *C*: STZ-administered double-transgenic mice showed food and fluid intake similar to healthy controls, whereas STZ-administered controls developed polydipsia and polyphagia. All results are the means ± SE of 12 mice in each group. *$P < 0.05$ vs. STZ-control.

adjusted to pH 5 with 5 mol/l K_2CO_3. Glycogen levels were measured using the α-amyloglucosidase method (22). For glucose tolerance testing, awake mice fasted overnight (16 h) were given an intraperitoneal injection of glucose (1 g/kg body wt). At the times indicated, blood samples were obtained from the tail vein of the same animals and the glucose concentration was measured. The pancreatic insulin release after a glucose load was determined in overnight-fasted mice by measuring the serum insulin levels after an intraperitoneal injection of 3 g/kg body wt of glucose.

Histological analysis. For immunohistochemical detection of insulin, pancreases were fixed for 24 h in formalin, embedded in paraffin, and sectioned. Sections were then incubated overnight at 4°C with a guinea pig anti-porcine insulin antibody (Dako, Carpinteria, CA) at 1:100 dilution. As secondary antibody, rabbit anti–guinea pig IgG, coupled to peroxidase (Roche Molecular Biochemicals), was used. DAB (3′3′-diaminobenzidine; Sigma, St. Louis, MO) was used as substrate chromogen. Sections were counterstained in Mayer's hematoxylin. Skeletal muscle expression of green fluorescent protein (GFP) was analyzed in toto in tibialis cranealis by fluorescence microscopy 1 week after vector administration. GFP expression in muscle sections was determined using a rabbit anti-GFP antibody (Molecular Probes, Leiden, the Netherlands) diluted at 1:500. As secondary antibody, biotinylated goat anti-rabbit antibody and ABC complex (Vector, Burlingame, CA) were used.

Statistical analysis. Serum parameters and metabolite concentrations are expressed as the means ± SE. The significance of differences was assessed, using the Student-Newmann-Keuls test. Differences were considered significant at $P < 0.05$.

RESULTS

Expression of insulin and glucokinase in skeletal muscle of double-transgenic mice prevented diabetic hyperglycemia. To examine whether insulin and glucokinase expressed in skeletal muscle may work in concert to reduce diabetic hyperglycemia, we crossed transgenic mice expressing insulin with those expressing glucokinase (16,17). Skeletal muscle of double-transgenic mice expressed high levels of both insulin and glucokinase genes 4 months after streptozotozin (STZ) administration (Fig. 1*A*, *upper panel*). This was concomitant with normal levels of GLUT4 protein in the plasma membrane (Fig. 1*A*, *bottom panel*). STZ-administered controls showed blood glucose levels of >600 mg/dl, the upper limit of measurement (Glucometer Elite; Bayer). In contrast to fed transgenic mice expressing glucokinase or insulin, which remained hyperglycemic 45 days after STZ administration (Fig. 1*B*), double Ins+GK–transgenic mice only showed a mild increase in blood glucose levels for 4 months after β-cell destruction (Fig. 1*B* and *C*). This was consistent with synergic action between the insulin produced by skeletal muscle and the increased glucose phosphorylation by glucokinase in preventing hyperglycemia. Furthermore, STZ-administered Ins+GK transgenic mice were normoglycemic after an overnight fast (Fig. 1*D*). Hypoglycemia was not observed when these mice were fasted for 24 h (Fig. 1*E*). This indicated that basal production of insulin by skeletal muscle provided the levels of insulin required to maintain normoglycemia between meals and that glucokinase's high K_m for glucose ensured that skeletal muscle did not increase glucose uptake when blood glucose levels were low. Compared with diabetic controls, which presented low levels of circulating insulin, fed transgenic mice showed increased insulinemia 4 months after STZ administration (Fig. 1*F*). This was consistent with skeletal muscle production of insulin. Furthermore, in contrast to fed STZ-administered controls that had reduced skeletal muscle glycogen content, fed STZ-administered Ins+GK transgenic mice showed glycogen levels similar to those of healthy controls: 2.15 ± 0.22 mg/g for controls, 1.25 ± 0.24 mg/g for STZ-administered controls, and 2.08 ± 0.22 mg/g for STZ-administered Ins+GK mice ($P < 0.05$ vs. STZ-administered controls), $n = 6$ per group. This finding suggests that increased glucose uptake by skeletal muscle was a primary mechanism leading to normalization of muscle glucose metabolism.

During diabetes, because of the lack of insulin, the hepatic expression of glucokinase, the main regulatory enzyme of glucose uptake, is low, whereas expression of PEPCK, a key regulatory enzyme of gluconeogenesis, is

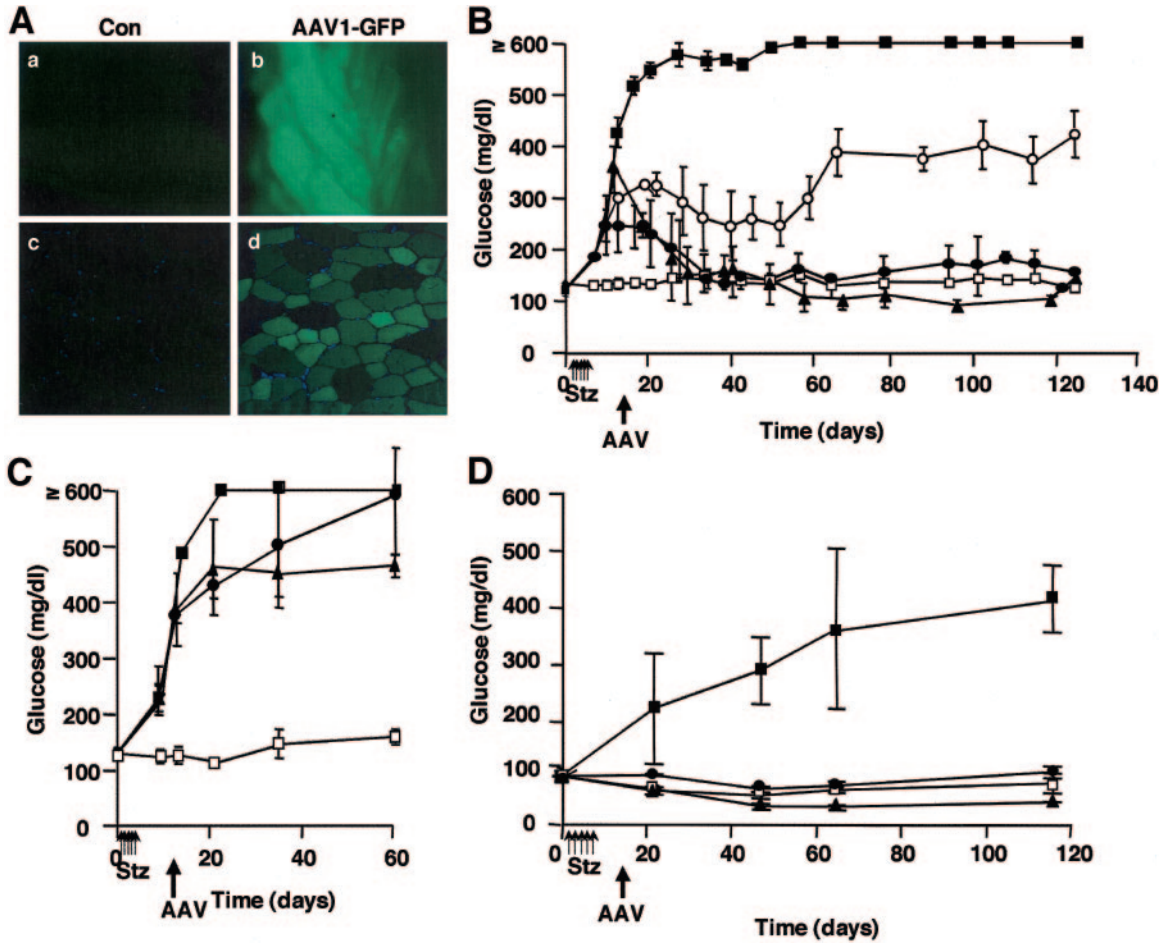

FIG. 3. AAV-mediated insulin and glucokinase coexpression normalized diabetic hyperglycemia. *A*: Expression of GFP in muscle fibers after treatment with 1×10^{10} vector genomes of AAV-1-GFP. In toto analysis showed high transduction of tibialis cranealis muscle (*a* and *b*). Sections of this muscle immunostained with a specific GFP antibody showed that ~80% of muscle fibers were transduced and expressed high levels of GFP (*c* and *d*). *B*: Fed blood glucose levels were determined in healthy control (□) and in STZ-administered control (■) and AAV1-Ins+GK–transduced (6×10^{10} [▲], 3.6×10^{10} [○], or 2.4×10^{10} [○] vector genomes of a mixture of 50% AAV1-Ins and 50% AAV1-GK) mice. *C*: Fed blood glucose levels were determined in healthy control (□), STZ-administered control (■), and AAV1-Ins–transduced (1.8×10^{10} [○] or 2.4×10^{10} [▲] vector genomes of AAV1-Ins) mice. *D*: Fasted blood glucose was determined in the animals indicated in *A*. All results are the means ± SE of 10 mice in each group. **P* < 0.05 vs. STZ-control. Con, control.

induced (23). The presence of circulating insulin led to an increase in glucokinase and a decrease in PEPCK gene expression in the liver of STZ-administered Ins+GK-expressing transgenic mice, which showed levels similar to those of healthy mice (Fig. 2*A*). This may have decreased hyperglycemia by blocking hepatic glucose production and increasing glucose uptake. In addition, serum concentrations of triglycerides, free fatty acids, and β-hydroxybutyrate (Fig. 2*B*) were normalized in fed STZ-administered Ins+GK-expressing transgenic mice. Consistent with all of these findings, STZ-administered Ins+GK-expressing transgenic mice presented normal fluid and food intake, whereas diabetic controls showed polyphagia and polydipsia (Fig. 2*C*). These results indicate that the expression of insulin and glucokinase in skeletal muscle of transgenic mice counteracts diabetic alterations, suggesting that it may be considered as a new approach for gene therapy for type 1 diabetes.

AAV1-mediated expression of insulin and glucokinase in skeletal muscle reversed diabetic alterations. To obtain long-term expression of Ins+GK genes, we next examined the use of AAV vectors to transduce skeletal muscle. It is now well established that the AAV1 serotype transduces murine skeletal muscle more efficiently than the more widely used AAV2, and it confers long-term gene expression (24–26). Thus, to obtain AAV1-GFP, AAV1-Ins, and AAV1-GK vectors, the cyotmegalovirus (CMV)/GFP, CMV/human proinsulin, or CMV/glucokinase gene was introduced into an AAV2-based vector that was packaged into an AAV1 capsid. When mouse tibialis cranialis muscle was injected with a single dose of 1×10^{10} viral genomes of AAV-1-GFP, ~80% of fibers expressed GFP (Fig. 3*A*). In these mice, no GFP expression was detected in the liver (data not shown).

When STZ-administered mice showed hyperglycemia (>300 mg/dl), a single vector dose of 1×10^{10}, 6×10^{9}, or 4×10^{9} vector genomes (50% AAV1-Ins and 50% AAV1-GK) was injected into tibialis cranealis, gastrocnemius, and quadriceps muscles of hindlimbs (total vector dose per mouse: 6×10^{10}, 3.6×10^{10}, or 2.4×10^{10} vector genomes). Although the dose of 2.4×10^{10} vector genomes only partially reduced hyperglycemia, doses of 3.6×10^{10} and 6×10^{10} vector genomes led to complete normalization of blood glucose levels in fed diabetic mice (Fig. 3*B*). These mice remained normoglycemic for >4 months. In contrast, fed diabetic mice treated only with AAV1-Ins vectors

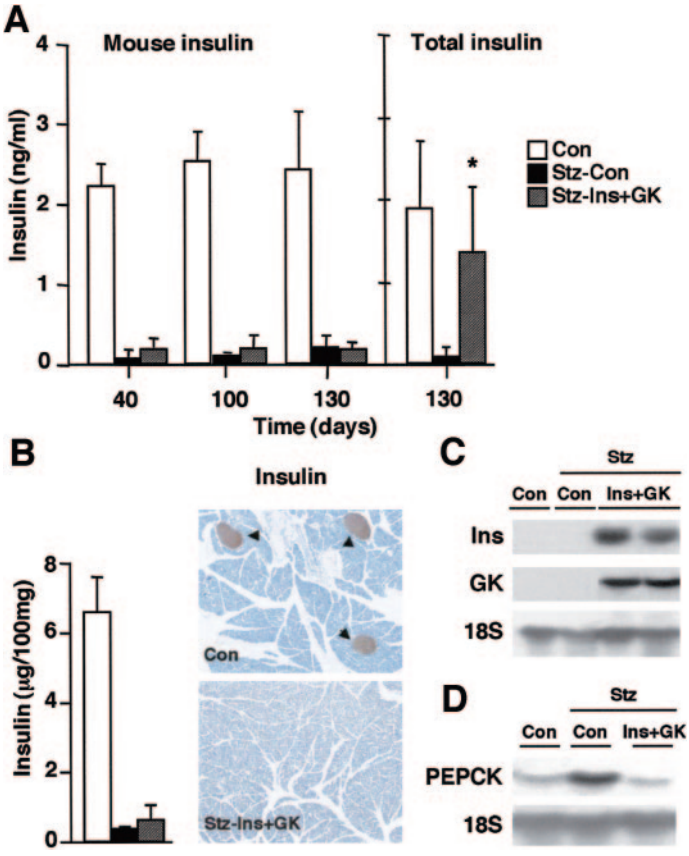

FIG. 4. Serum, pancreas, and muscle insulin in AAV-treated mice. *A*: Serum insulin concentration. Mouse insulin concentration was low in all STZ-administered mice. Increased levels of total insulin (mouse plus human) were noted in STZ-administered mice that received a single injection of AAV-Ins+GK vectors (3.6×10^{10} vector genomes). *B*: Pancreatic insulin content and insulin immunostaining in islets of pancreatic sections were greatly reduced 4 months after STZ administration. Arrowheads indicate mouse islets (×100). Expression of insulin and glucokinase (*C*) in skeletal muscle and hepatic expression of PEPCK (*D*) 4 months after a single injection of AAV-Ins+GK vectors (3.6×10^{10} vector genomes). Total RNA was obtained from either skeletal muscle or liver, analyzed by Northern blot, and hybridized with insulin, glucokinase, or PEPCK probes. A representative Northern blot is shown. All results are the means ± SE of 10 mice in each group. *$P <$ 0.05 vs. STZ-control. Con, control; GK, glucokinase; Ins, insulin.

(1.8×10^{10} and 2.4×10^{10} vector genomes) developed hyperglycemia (Fig. 3*C*). In fasted conditions, all diabetic mice treated with Ins+GK-AAV1 vectors were normoglycemic (Fig. 3*D*). However, diabetic mice treated with the 6×10^{10} vector genomes showed a tendency to develop hypoglycemia in fasted conditions, which indicated that the level of expression of the insulin and glucokinase was probably too high. Injection of 3.6×10^{10} vector genomes of AAV vectors was more appropriate because hypoglycemia was not observed. All subsequent studies were performed with this dose of vectors.

Circulating mouse insulin levels were low in all diabetic mice (Fig. 4*A*). This was consistent with the finding that STZ-administered mice showed a reduction (~90%) of pancreatic insulin content, and insulin-producing β-cells were hardly detected in pancreatic islets (Fig. 4*B*). In contrast, skeletal muscle of these AAV-transduced diabetic mice showed high levels of insulin and glucokinase gene expression (Fig. 4*C*). Furthermore, Ins+GK-treated diabetic mice showed high levels of circulating total insulin, which resulted from the human insulin released by the skeletal muscle (Fig. 4*A*). Moreover, whereas no human C-peptide was detected in serum of STZ-administered control mice, STZ-administered Ins+GK-expressing transgenic mice presented levels of 0.85 ± 0.21 ng/ml (n = 5), indicating that mature human insulin was produced by the engineered muscles. This restored PEPCK (Fig. 4*D*) and glucokinase (data not shown) gene expression in the liver of these diabetic mice. In diabetic Ins+GK-expressing transgenic mice, serum triglyceride concentration was also similar to that of nondiabetic controls (data not shown), which was consistent with the normalization of hepatic metabolism. Furthermore, in contrast to diabetic nontreated mice, Ins+GK-expressing transgenic diabetic mice showed normal glucose tolerance, indicating that they responded to an intraperitoneal glucose load similarly to healthy mice (Fig. 5*A*). Pancreatic insulin release was also determined in overnight-fasted mice after intraperitoneal injection of glucose (3 g/kg body wt). Serum levels of mouse insulin remained very low in both nontreated and Ins+GK-treated diabetic mice, which was consistent with the lack of β-cells (Fig. 5*B*). Moreover, the normalization of glycemia was parallel to normalization of fluid and food intake (Fig. 5*C*).

During type 1 diabetes, the lack of insulin leads to increased protein breakdown in skeletal muscle, release of amino acids as gluconeogenic substrates, and lipolysis in adipose tissue. Diabetic control mice showed a marked weight reduction in skeletal muscle (~30%) and abdominal fat (~70%). In contrast, Ins+GK-treated diabetic mice had normal weight for these tissues 4 months after STZ administration (Fig. 5*D*). Furthermore, histological analysis revealed that skeletal muscles of Ins+GK-treated mice were normal (data not shown). In addition, the appearance of the abdominal organs of Ins+GK-treated diabetic mice was similar to that of healthy mice, whereas nontreated diabetic controls showed marked distension of intestinal loops and absence of abdominal fat (Fig. 5*E*). All of these results indicate that skeletal muscle expression of insulin and glucokinase genes by using AAV1 vectors led to long-term normalization of glucose homeostasis and reversed diabetic alterations.

DISCUSSION

The main goal of any treatment of type 1 diabetes is to maintain normoglycemia. The findings of our study demonstrate for the first time that skeletal muscle, the key tissue in glucose uptake, can be engineered to regulate whole-body glucose homeostasis in diabetic mice. This was achieved by generating a glucose sensor as a result of the synergistic action of two genes, insulin and glucokinase. One of the main concerns in non–β-cell insulin production is the difficulty of releasing the right amount of insulin when it is needed. Pancreatic β-cells are highly sensitive to blood glucose levels, and insulin secretion is rapidly triggered in response to any change (27). It is difficult to reproduce such efficient regulation by engineering extrapancreatic tissue. Although many advances in hepatic insulin gene therapy have been made over the past few years, precise regulation of insulin production and secretion is still a major challenge (7,28,29). In our approach, insulin was constitutively expressed at basal levels in skeletal muscle of diabetic mice, which allowed the maintenance of normoglycemia between meals. Mice expressing insulin and glucokinase after treatment with viral vectors showed normoglycemia, indicating that enough insulin was produced by the skeletal muscle to supply the

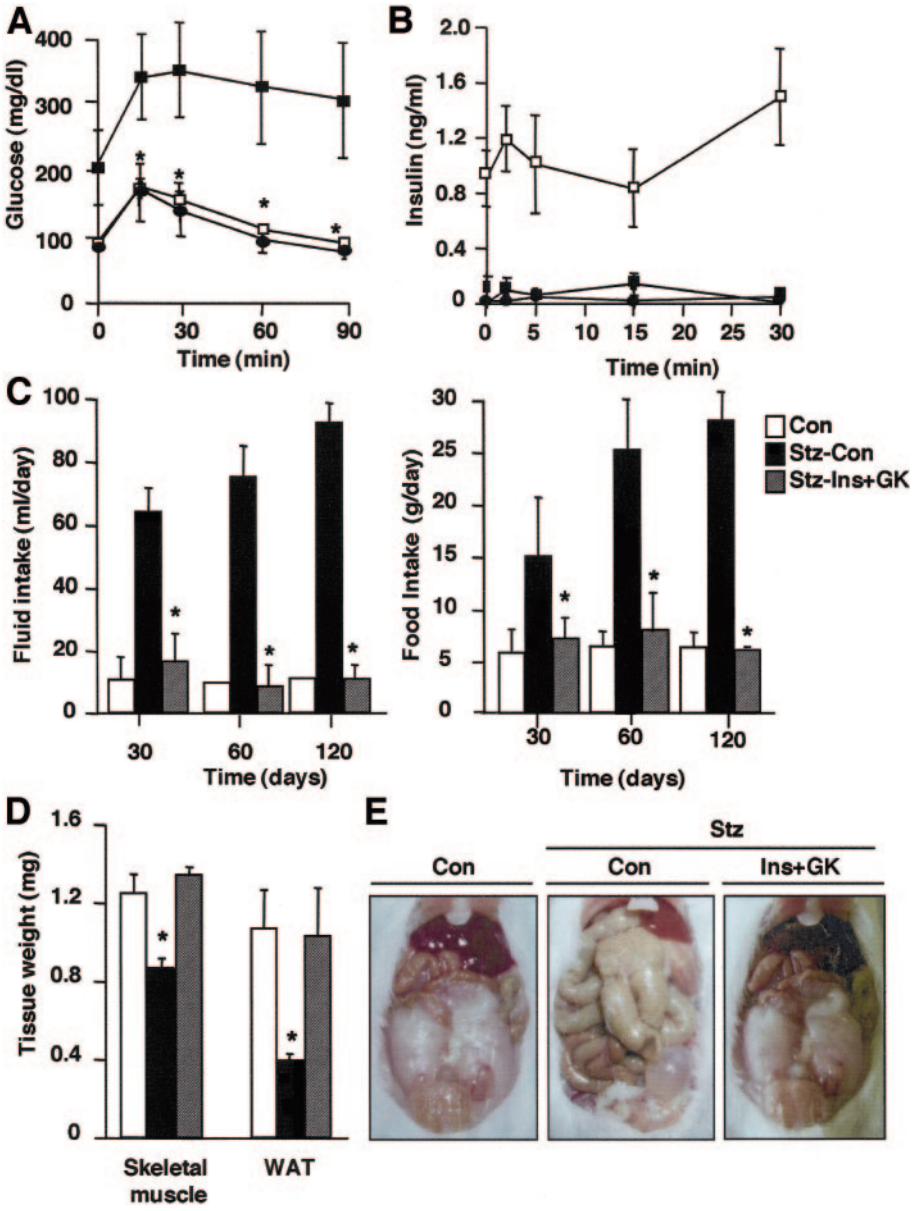

FIG. 5. Long-term expression of insulin and glucokinase prevented diabetic alterations. *A*: At 3 months after STZ administration, AAV-Ins+GK–transduced mice showed normal glucose tolerance. Mice were given an injection of 1 mg glucose/g body wt i.p. *B*: At 4 months after STZ administration, AAV-Ins+GK–transduced mice did not show pancreatic insulin release after a glucose load. Mice were given an injection of 3 mg glucose/g body wt i.p., and mouse insulin levels were measured at the indicated times. *C*: STZ-administered AAV-Ins+GK–transduced (3.6×10^{10} vector genomes) mice develop neither polydipsia nor polyphagia. Fluid and food intake were determined in individual animals at the indicated times after STZ administration. All of these results (*A–C*) are the means ± SE of 10 mice in each group. *$P < 0.05$ vs. STZ-control. *D*: The weight of the skeletal muscle and of the abdominal fat pad was preserved in AAV-Ins+GK–transduced (3.6×10^{10} vector genomes) mice 4 months after STZ administration. Results are the means ± SE of 7 mice in each group. *$P < 0.05$ vs. control healthy mice. *E*: The abdominal organ appearance of AAV-Ins+GK–treated diabetic mice was normal 4 months after STZ administration. Nontreated diabetic controls showed marked distension of intestinal loops and absence of abdominal fat. Overnight-fasted healthy control (□), STZ-administered control (■), and AAV-Ins+GK–transduced (3.6×10^{10} vector genomes) (●) mice. Con, control.

whole-body demand during fasting without inducing hypoglycemia. Furthermore, because glucokinase has a high K_m for glucose (12), it prevented glucose uptake when blood levels were reduced, thus contributing to the maintenance of normoglycemia. Moreover, normoglycemia was also achieved in fed conditions in Ins+GK-expressing transgenic animals, which did not occur when skeletal muscle was transduced with AAV1-Ins. Similarly, fed diabetic transgenic mice expressing either insulin or glucokinase remain hyperglycemic (16,30). Neither overexpression of GLUT4 in skeletal muscle of STZ-administered transgenic mice nor overexpression of GLUT4 plus hexokinase II leads to normalization of hyperglycemia, probably due to decreased GLUT4 translocation to plasma membrane in the absence of insulin and hexokinase II inhibition by glucose 6-phosphate (31). In our approach, since GLUT4 expression in plasma membrane was restored by the basal insulin production, normalization of glycemia mainly resulted from glucose-regulated glucose phosphorylation by glucokinase, which increased glucose utilization in skeletal muscle. Furthermore, the presence of circulating insulin, together with decreased glycemia, led to an increase in glucokinase and a decrease in PEPCK gene expression in the liver of Ins+GK-expressing transgenic mice. This fact may have increased glucose uptake and decreased glucose production by the liver, thus contributing to the maintenance of normoglycemia. In contrast, normoglycemia in both fed and fasted conditions as well as normal response to glucose tolerance tests are difficult to achieve by approaches based only on transcriptionally regulated systems for insulin production, such as the use of liver-specific promoters that respond to the complex interaction of glucose, insulin, and other hormones (32–35).

Furthermore, AAV vectors have proven to be very useful to deliver sustained expression of key genes for the treatment of several diseases in mice, larger animals, and humans (36,37). Here, we found that a single injection of AAV1 vectors carrying insulin and glucokinase genes normalized glycemia for >4 months. Widespread transduction of skeletal muscle was achieved without any toxic response at the doses of vector used (6×10^{10} vector

genomes). Greater intramuscular doses of AAV have been administered to larger animals and also in humans with no adverse effects (36,38,39).

In summary, we report evidence that a novel gene therapy approach provides blood glucose concentration–dependent regulation of glucose homeostasis in diabetic animals. The significance of our results is that, since normalization of glycemia is achieved, secondary complications of type 1 diabetes will not develop. This is certain to improve the quality of life of diabetic patients. Furthermore, because this approach is based on engineering skeletal muscle, it has several advantages (tissue accessibility, no need for immunosuppression) over other approaches, such as engineering the liver or transplanting insulin-producing β-cells (5,6). Thus, the concept developed in our study by combining the effects of two genes working synergistically may represent a major advance in the treatment of diabetes. Nevertheless, extensive studies in larger animal models of diabetes are needed to determine efficacy, long-term effects, safety, and feasibility before such an approach may be applied to human patients.

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