Counteraction of Type 1 Diabetic Alterations by Engineering Skeletal Muscle to Produce Insulin

Insights From Transgenic Mice

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Insulin replacement therapy in type 1 diabetes is imperfect because proper glycemic control is not always achieved. Most patients develop microvascular, macrovascular, and neurological complications, which increase with the degree of hyperglycemia. Engineered muscle cells continuously secreting basal levels of insulin might be used to improve the efficacy of insulin treatment. Here we examined the control of glucose homeostasis in healthy and diabetic transgenic mice constitutively expressing mature human insulin in skeletal muscle. Fed transgenic mice were normoglycemic and normoinsulinemic and, after an intraperitoneal glucose tolerance test, showed increased glucose disposal. When treated with streptozotocin (STZ), transgenic mice showed increased insulinemia and reduced hyperglycemia when fed and normoglycemia and normoinsulinemia when fasted. Injection of low doses of soluble insulin restored normoglycemia in fed STZ-treated transgenic mice, while STZ-treated controls remained highly hyperglycemic, indicating that diabetic transgenic mice were more sensitive to the hypoglycemic effects of insulin. Furthermore, STZ-treated transgenic mice presented normalization of both skeletal muscle and liver glucose metabolism. These results indicate that skeletal muscle may be a key target tissue for insulin production and suggest that muscle cells secreting basal levels of insulin, in conjunction with insulin therapy, may permit tight regulation of glycemia. *Diabetes* 51:704–711, 2002

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2-DG, 2-[1-^{3}H]deoxy-D-glucose; DAB, diaminobenzidine; FFA, free fatty acid; GK, glucokinase; HKII, hexokinase II; RIA, radioimmunoassay; STZ, streptozotocin; Tg, transgenic line.

Type 1 diabetes generally appears during youth and results from autoimmune destruction of insulin-producing cells in the pancreas (1,2). Patients depend dramatically on insulin replacement therapy, which, although it allows them to lead active lives, is imperfect and strongly affects their lifestyle. Chronic hyperglycemia due to failure to maintain proper glycemic control leads to the development of diabetes-specific microvascular pathology in the retina and renal glomerulus and neurological and macrovascular complications (3). Intensive insulin therapy can delay the onset and slow the progression of microvascular complications (4). However, the current therapy for most diabetic patients is based on daily subcutaneous injections of mixtures of soluble (short-acting) insulin and lente insulin preparations. Suspensions of soluble insulin particles of different size that give intermediate-acting and long-acting components with more sustained action profiles are administered to achieve a constant basal level of the hormone (5). However, one of the major deficiencies of delayed-action insulin is the variable absorption from subcutaneous tissue (6), mainly because the formulation is a suspension. Moreover, the delayed-action preparations available do not generally produce smooth background levels of insulin, resulting in either hyperglycemia or hypoglycemia.

In addition to attempts to produce new, very long–acting insulin analogs, pancreas and islet transplantation have also been used as alternatives for restoring endogenous insulin secretion (7). However, the limited availability of pancreatic tissue donors and the potential autoimmune reactions are severe restrictions. Thus, research based on the use of surrogate cells to deliver insulin is strongly emerging. Approaches centered on developing and transplanting cell lines derived from β-cells or neuroendocrine cells (8–10), insulin-secreting cells derived from ES-cells (11), islets generated in vitro from pancreatic stem cells (12), and also non–β-cells, such as hepatocytes (13–16), muscle (17), and fibroblast cells (18), are being used in pursuit of this goal. In addition, in vivo approaches have been developed to induce the liver to express regulated insulin (19–21). However, to obtain a more feasible approach to diabetes gene therapy, skeletal muscle might be engineered to secrete insulin, since it has the advantage of accessibility. Moreover, it has been demonstrated that skeletal muscle is an excellent target tissue for

expression of secretory proteins in several gene therapy approaches (22,23).

Skeletal muscle has been estimated to account for ~60–75% of glucose uptake after a meal. Insulin-stimulated glucose transport is performed by GLUT4 (24,25). Glucose is rapidly converted to glucose-6-phosphate by hexokinase II (HKII) (26,27), the insulin-stimulated hexokinase isoform. GLUT4 translocation to plasma membrane and HKII mRNA levels and activity decrease when circulating insulin is low (28,29). In the therapy of diabetes by engineering skeletal muscle to produce insulin, the synthesized hormone may be released to the blood and also may induce skeletal muscle to take up glucose by acting in a paracrine/autocrine manner. Local production of insulin may lead to the presence of GLUT4 in the plasma membrane and increased HKII activity and, thus, to higher blood glucose disposal. In the present study, we used a transgenic animal model to study the feasibility of using the skeletal muscle as a source of basal constant release of insulin for the gene therapy of diabetes. Furthermore, we attempted to determine the effects of long-term production of the hormone on healthy and diabetic animals. To this end, we obtained transgenic mice carrying a chimeric gene obtained by linking the myosin-light chain 1 promoter to the human proinsulin gene, containing genetically engineered furin endoprotease cleavage sites (MLC1/Insm) (17).

RESEARCH DESIGN AND METHODS

Generation of transgenic mice. The MLC/Insm chimeric gene used to obtain transgenic mice was previously described (17). A 3.4-kb *Asp*718-*Sac*I fragment, containing the entire MLC/Insm chimeric gene was microinjected into fertilized eggs. The general procedures for microinjection of the chimeric gene were as described (30). At 3 weeks of age, the animals were tested for the presence of the transgene by PCR analysis using primers previously described (17) and also by Southern blot of 10 μg of tail DNA digested with *Hin*dIII. Blots were hybridized with a 0.4 kb *Eco*RI-*Eco*RI fragment containing the mutated proinsulin cDNA radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) by random oligopriming (Roche Molecular Biochemicals, Mannheim, Germany).

Treatment of mice. Heterozygous male mice (C57Bl6/SJL) aged 2–4 months were fed ad libitum with a standard diet (Panlab, Barcelona, Spain) and maintained under a light-dark cycle of 12 h (lights on at 8:00 A.M.). When stated, mice were fasted for 16 h. Animals were killed and samples (quadriceps and gastrocnemius muscles and liver) were taken between 9:00 and 11:00 A.M. To induce insulin-dependent diabetes, mice were given, on five consecutive days, an intraperitoneal injection of streptozotocin (STZ) (50 mg/kg), dissolved in 0.1 mol/l citrate buffer (pH 4.5) immediately before administration. Diabetes was assessed by measuring blood glucose levels. All experimental procedures involving mice were approved by the Ethics and Experimental Animal Committee of the Autonomous University of Barcelona.

RNA analysis. Total RNA was obtained from skeletal muscle by the guanidine isothiocyanate method (31), and RNA samples (30 μg) were electrophoresed on a 1% agarose gel containing 2.2 mol/l formaldehyde. Northern blots were hybridized to the following ^{32}P-labeled probes: a 0.4-kb *Eco*RI-*Eco*RI fragment corresponding to proinsulin cDNA, a 4.2-kb *Eco*RI-*Not*I fragment corresponding to furin cDNA, a 2.7-kb *Eco*RI-*Eco*RI fragment corresponding to HKII cDNA, a 2.5-kb *Eco*RI-*Eco*RI fragment corresponding to GLUT4 cDNA, a 2.6-kb *Eco*RI-*Eco*RI fragment corresponding to GLUT1 cDNA, and a 1.3-kb *Eco*RI-*Eco*RI fragment corresponding to rabbit β-actin cDNA. These probes were labeled with [α-^{32}P]dCTP, following the method of random oligopriming as described by the manufacturer (Roche Molecular Biochemicals). Specific activity of the DNA probe thus labeled was ~10^9 cpm/μg DNA. Membranes were placed in contact with Kodak XAR-5 films (Rochester, NY). The β-actin signal was used to correct for loading inequalities.

HPLC analysis and measurement of insulin in muscle and pancreas extracts and in serum. Insulin concentrations in serum and in pancreas extracts were determined by radioimmunoassay (RIA) (CIS Biointernational, Guf-Sur-Yvetle, France) following the supplier's protocol, using specific ^{125}I-labeled porcine insulin as tracer and antibody that is 100% crossreactive with human insulin, 90% crossreactive with rat insulin, and <14% crossreactive with human proinsulin. Serum proinsulin was also determined by RIA (Linco Research, St. Charles, MO) following the manufacturer's instructions, using specific ^{125}I-labeled human proinsulin as tracer and antibody that does not crossreact with insulin. To determine pancreatic insulin content, whole pancreata were removed from the mice, weighed, and homogenized in 20 volumes of cold acidic ethanol (75% ethanol, 1.5% concentrated HCl) followed by 48 h of agitation at 4°C. Afterward, insulin was quantified in the supernatants of the samples by RIA. To determine proinsulin processing, HPLC (Waters, Milford, MA) was performed on acid extracts of skeletal muscle. Briefly, frozen hindlimbs of control and transgenic mice were weighed and homogenized 1:5 (wt/vol) in 1 mol/l acetic acid using a tissue homogenizer. Each muscle homogenate was centrifuged at 600*g* for 15 min. The supernatant fraction was lyophilized and reconstituted in 0.5× volume with 50 mmol/l Tris-HCl (pH 7.8) and then clarified by centrifugation at 3,000*g* for 10 min. Samples were then loaded on a reverse-phase C18 column (μbondapack, Waters PN 27324), protected by a precolumn of C18 Corasil and equilibrated with 0.1% trifluoroacetic acid (pH 2.0), and eluted with a linear gradient of 20–50% acetonitrile for 30 min at 1.5 ml/min. Fractions were collected every 30 s for 10 min. These fractions were lyophilized and resolved in 100 μl of 0.1 mol/l borate buffer, pH 8.6, containing 0.5% BSA. Samples were then analyzed by RIA, using an insulin polyclonal antibody characterized previously (32). This insulin antibody recognized proinsulin up to 90%. The HPLC system was calibrated using a mixture containing 5×10^{-4} mol/l porcine insulin and 5×10^{-5} mol/l human recombinant proinsulin (Sigma Chemical, St. Louis, MO).

Histological analysis. For immunohistochemical detection of insulin, pancreata from control and transgenic mice were fixed for 12–24 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 a secondary antibody, rabbit anti–guinea pig immunoglobulin G, coupled to peroxidase (Boehringer Mannheim), was used. 3′3′-diaminobenzidine (DAB) (Sigma, St. Louis, MO) was used as substrate chromogen. Sections were counterstained in Mayer's hematoxylin.

Enzyme and metabolite assays. To determine enzyme activities and the concentration of metabolites, skeletal muscle and liver samples were clamped, frozen in situ, and kept at −80°C until analysis. Hepatic glucokinase (GK) activity was analyzed in liver samples as previously described (33). The concentrations of glycogen, glucose-6-phosphate, and lactate were measured in perchloric extracts, which were adjusted to pH 5 with 5 mol/l K_2CO_3 to determine glycogen and to pH 7 for glucose-6-phosphate and lactate. Glycogen levels were measured using the α-amyloglucosidase method (34). Glucose-6-phosphate was determined enzymatically (35). Lactate was measured by the lactate dehydrogenase method (Roche Molecular Biochemicals). Glucose levels in serum were determined enzymatically (Glucoquant; Roche Molecular Biochemicals). Glucose concentration in blood was determined by using a Glucometer Elite (Bayer, Farrytown, Germany) following the manufacturer's instructions. Serum free fatty acids (FFAs) were measured by the acyl-CoA synthase and acyl-CoA oxidase method (Wako Chemicals, Ness, Germany). The β-hydroxybutyrate levels in serum were measured by the β-hydroxybutyrate dehydrogenase technique (Roche Molecular Biochemicals). Serum triglycerides were determined enzymatically (GPO-PAP; Roche Molecular Biochemicals).

In vivo glucose utilization index. An introvenous flash injection of 1 μCi of the nonmetabolizable glucose analog 2-[1-^{3}H]deoxy-D-glucose (2-DG) (Amershan Pharmacia Biotech) was administered to fed mice. The specific blood 2-DG clearance was determined using the Somogogy procedure (36) with 25 μl blood samples (tail vein) obtained 1, 10, 20, and 30 min after injection. Skeletal muscle samples (gastrocnemious and quadriceps) were removed 30 min after injection. The glucose utilization index was determined by quantitating the accumulation of radiolabeled compounds using a previously validated method (37). The amount of 2-DG-6-phosphate per milligram of protein was divided by the integral of the concentration ratio of 2-DG to unlabeled glucose measured. Because values were not corrected by a "discrimination constant" for 2-DG in glucose metabolic pathways, the results were expressed as the index of glucose utilization in picomoles per milligram of protein per minute.

Statistical analysis. Enzyme activities 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 human insulin in skeletal muscle. The MLC/Insm chimeric gene was microinjected into fertilized eggs, and three lines of transgenic mice were obtained.

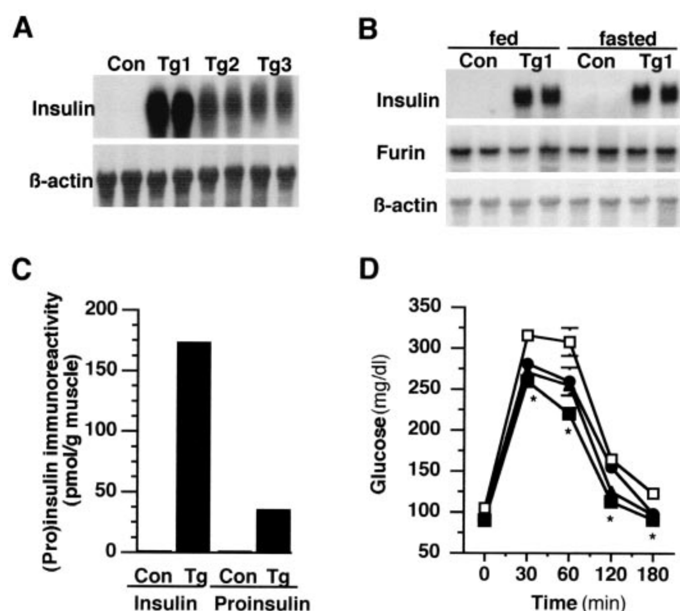

FIG. 1. Expression of human insulin in skeletal muscle of transgenic mice. *A*: Total RNA was obtained from hindlimb skeletal muscle (quadriceps and gastrocnemius) of fed control (Con) and transgenic mice from lines MLC/Insm-1 (Tg1), MLC/Insm-2 (Tg2), and MLC/Insm-3 (Tg3) and analyzed as indicated in RESEARCH DESIGN AND METHODS. A representative Northern blot hybridized with insulin is shown. *B*: Insulin and furin gene expression in skeletal muscle of fed and fasted control (Con) and Tg1 transgenic mice. A representative Northern blot hybridized with insulin, furin, and β-actin cDNA probes is shown. *C*: Insulin and proinsulin immunoreactivity products in skeletal muscle. HPLC and RIA analyses were performed in perchloric acid extracts of skeletal muscle obtained from fed control and Tg1 transgenic mice, as indicated in RESEARCH DESIGN AND METHODS. Results are means ± SE of six different animals in each group. *D*: Intraperitoneal glucose tolerance test. Overnight-starved transgenic Tg1 (■), Tg2 (●), Tg3 (▲), and control mice (□) were given an injection of 1 mg i.p. of glucose per gram body weight. At the times indicated, blood samples were taken from the tail vein of the same animals. Glucose was determined as indicated in RESEARCH DESIGN AND METHODS. Results are means ± SE of 10 transgenic and 10 control mice (P* < 0.05 Tg1 vs. Con).**

Transgenic line 1 (Tg1) was estimated to carry approximately four intact copies of the MLC/Insm chimeric gene, transgenic line 2 (Tg2) carried ~10, and transgenic line 3 (Tg3) carried ~15 when analyzed by Southern blot (data not shown). The MLC1 promoter/enhancer directs the expression of foreign genes specifically to skeletal muscle (selective expression in fast-twitch muscle fibers), in a rostrocaudal gradient (39,40). The three lines of transgenic mice expressed insulin mRNA in skeletal muscle (quadriceps and gastrocnemius), while no insulin mRNA was noted in controls (Fig. 1*A*). No expression of the transgene was detected in other tissues of transgenic mice, like liver or spleen (data not shown). Fed Tg1 transgenic mice showed higher levels (approximately threefold) of insulin mRNA in skeletal muscle than Tg2 and Tg3 (Fig. 1*A*), which presented similar level of expression of the transgene. Moreover, Tg1 mice had similar insulin gene expression in both fed and fasted conditions, consistent with constitutive expression of the transgene from the MLC1 promoter (Fig. 1*B*). Furthermore, a similar level of furin mRNA was noted in skeletal muscle of fed and fasted control and transgenic mice (Fig. 1*B*), suggesting that muscle cells might process proinsulin to mature insulin in vivo. Each experiment described below was mainly performed in F1 and F2 generations from Tg1 heterozygous transgenic mice, which expressed high levels of the transgene. We also studied Tg2 and Tg3 mice, which expressed low levels of the transgene. We used littermates as controls. Transgenic mice were healthy and had a normal lifespan and reproductive life.

TABLE 1
Blood glucose and serum insulin levels

	Glucose (mg/dl)	Insulin (μU/ml)
Fed		
Control	154 ± 9	35 ± 3
Tg1	147 ± 7	38 ± 4
Tg2	160 ± 8	36 ± 5
Tg3	145 ± 7	37 ± 4
Fasted		
Control	93 ± 6	14 ± 1
Tg1	75 ± 5*	18 ± 1*
Tg2	88 ± 5	14 ± 2
Tg3	91 ± 6	15 ± 3

Data are means ± SE of 10 animals in each group. Blood glucose and serum insulin from fed and fasted control and Tg1, Tg2, and Tg3 transgenic mice were determined as described in RESEARCH DESIGN AND METHODS. **P* < 0.05 vs. control.

Production of human insulin in skeletal muscle led to increased glucose disposal. The presence of immunoreactive insulin was determined in Tg1 mice after HPLC analysis of skeletal muscle extracts. Each HPLC fraction was subjected to RIA, and no immunoreactive products were identified in the skeletal muscle of control mice. In contrast, only two peaks were identified in muscle extracts of Tg1 mice, which corresponded to insulin and proinsulin according to their elution times (data not shown). A major peak corresponding to insulin (~85%) and a minor peak corresponding to proinsulin (~15%) were detected (Fig. 1*C*), indicating that proinsulin was highly processed to mature insulin by furin.

Insulin production by skeletal muscle of fed Tg1 mice did not significantly modify neither insulinemia (Table 1 and Fig. 2*A*) nor proinsulinemia (control 6.0 ± 0.5 μU/ml vs. transgenic 6.5 ± 0.3 μU/ml, *P* < 0.05). Similarly, fed Tg1 mice were normoglycemic (Table 1 and Fig. 2*C*). However, fasted Tg1 mice showed increased insulinemia (~28%) compared with controls (Table 1 and Fig. 2*B*). In addition, fasted Tg1 mice showed increased levels of proinsulin (~50%) compared with controls (control 2 ± 0.2 μU/ml vs. transgenic 3 ± 0.2 μU/ml, *P* < 0.05). This was concomitant with a reduction (~20%) of blood glucose concentration (Table 1 and Fig. 2*D*). In contrast, Tg2 and Tg3 transgenic mice were normoinsulinemic and normoglycemic in both fed and fasted conditions (Table 1). This suggested that these mice had lower production of insulin by skeletal muscle than Tg1 mice. In addition, Tg1, Tg2, and Tg3 transgenic mice showed normal levels of serum triglycerides, ketone body, cholesterol, and FFAs in both fed and fasted conditions (data not shown).

When an intraperitoneal glucose tolerance test was performed in overnight-fasted mice, blood glucose levels in Tg1 mice were lower (~40%) than those of controls (Fig. 1*D*), suggesting that insulin production by the skeletal muscle caused an increase in glucose disposal. Tg2 and Tg3 mice only showed a small increase in blood glucose disposal compared with Tg1 mice (Fig. 1*D*). Furthermore, a significant increase (~60%) in the glucose utilization

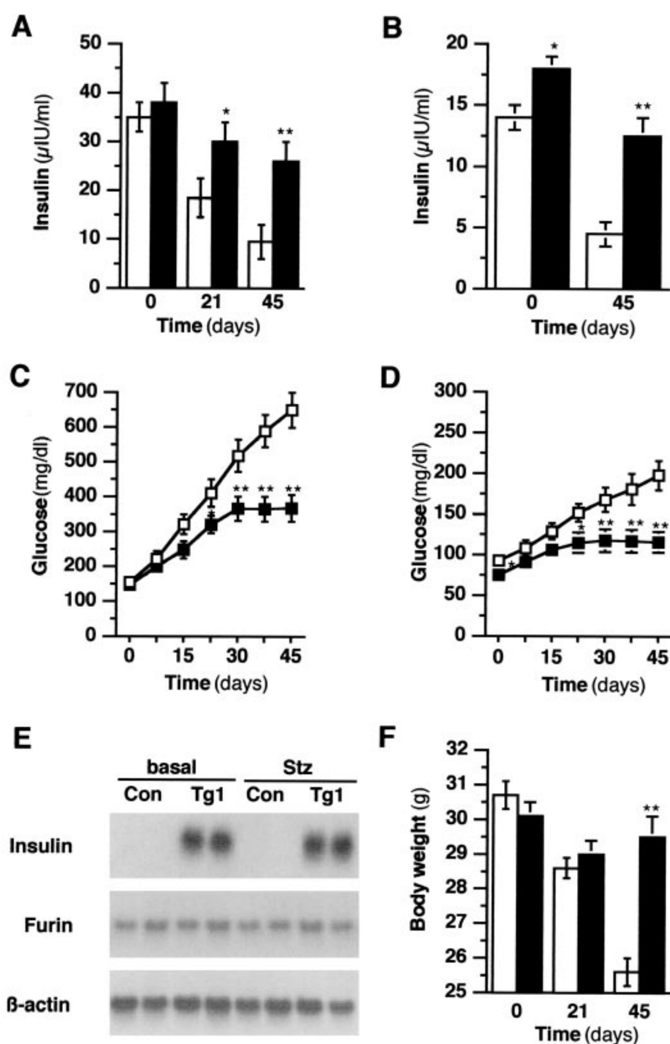

FIG. 2. Serum insulin (*A* and *B*) and glucose levels (*C* and *D*) were measured either in fed (*A* and *C*) or in overnight-fasted (*B* and *D*), control (□) and Tg1 transgenic (■) mice after STZ treatment. Serum insulin and glucose levels were measured as described in RESEARCH DESIGN AND METHODS. *E*: Expression of the transgene in skeletal muscle of STZ-treated mice (Stz). Total RNA was obtained from skeletal muscle of control (Con) and Tg1 transgenic mice and analyzed as indicated in RESEARCH DESIGN AND METHODS. A representative Northern blot hybridized with insulin, furin, and β-actin cDNA probes is shown. *F*: Body weight changes in control (□) and Tg1 transgenic (■) mice 21 and 45 days after STZ treatment are shown. Results are mean ± SE of 10 mice in each group (*P < 0.05 vs. Stz-Con, **P < 0.01 vs. Stz-Con).

index was detected in skeletal muscle (quadriceps and gastrocnemius) of Tg1 transgenic mice compared with controls (267 ± 31 pmol · mg^{-1} protein · min^{-1} for control vs. 430 ± 38 pmol pmol · mg^{-1} protein · min^{-1} for Tg1 mice ($P < 0.05$)). Therefore, the expression of insulin in skeletal muscle led to higher glucose utilization.

When the pancreatic insulin content was measured, similar concentration of the hormone was noted in both Tg1 mice and controls (control 6.1 ± 0.4 μg insulin/100 mg pancreas vs. Tg1 5.8 ± 0.3 μg insulin/100 mg pancreas, $P <$ 0.05). These results suggested that insulin produced by the skeletal muscle of Tg1 transgenic mice did not alter pancreatic insulin content. Furthermore, the number and size of islets from control and Tg1 mice were the same (data not shown).

Reduction of diabetic hyperglycemia by skeletal muscle production of insulin. To determine whether the production of insulin in the skeletal muscle led to reduction of diabetic hyperglycemia, control and Tg1 mice were intraperitoneally injected with low doses (50 mg/kg body wt) of STZ for 5 consecutive days. Forty-five days after STZ-treatment, mice showed a high reduction (~88%) of pancreatic insulin content (0.75 μg insulin/100 mg pancreas). Skeletal muscle from both non–STZ-treated and STZ-treated Tg1 mice presented similar levels of insulin mRNA (Fig. 2*E*), indicating that STZ treatment did not alter the expression of the transgene. Similarly, furin gene expression was preserved in both control and Tg1 mice after STZ treatment (Fig. 2*E*).

Forty-five days after STZ-treatment, fed control mice had low levels of circulating insulin, while Tg1 mice only presented ~30% decrease in insulinemia (Fig. 2*A*). During starvation, STZ-treated Tg1 mice showed 15% reduction in insulinemia compared with fasted non–STZ-treated control mice, while STZ-treated controls showed a marked decrease (Fig. 2*B*). These results suggest that skeletal muscle production of insulin counteracted hypoinsulinemia in STZ-treated mice. Furthermore, fed STZ-treated control mice were highly hyperglycemic while fed STZ-treated Tg1 mice only showed a 2.5-fold increase in glycemia, indicating that insulin produced by skeletal muscle partially counteracted hyperglycemia (Fig. 2*C*). Moreover, while fasted STZ-treated controls increased glycemia twofold, fasted STZ-treated transgenic mice showed similar blood glucose levels to fasted non–STZ-treated controls (Fig. 2*D*). Hypoglycemia was not detected in fasted STZ-treated Tg1 mice. These findings indicate that the insulinemia noted in STZ-treated Tg1 mice was enough to maintain normoglycemia during starvation. Moreover, STZ-treated control mice showed reduced (~20%) body weight, while STZ-treated Tg1 mice maintained body weight (Fig. 2*F*). In addition, the concentration of serum triglycerides was normalized in fed STZ-treated Tg1 mice (Table 2). These mice also showed a marked reduction in serum β-hydroxybutyrate and FFA concentrations, which were increased in STZ-treated controls (Table 2). However, after STZ treatment of Tg2 and Tg3 mice, hyperglycemia was unchanged (data not

TABLE 2
Serum parameters in STZ-treated MLC/Insm transgenic mice

Parameter	Con	STZ-Con	STZ-Tg
β-Hydroxybutyrate (mmol/l)	0.7 ± 0.1	1.61 ± 0.2	0.94 ± 0.2*
Triglycerides (mg/dl)	160 ± 17	244 ± 15	156 ± 11*
Nonesterified fatty acids (mmol/l)	0.85 ± 0.1	1.43 ± 0.1	0.98 ± 0.2*

Data are means ± SE of 10 animals in each group. β-hydroxybutyrate, triglycerides, FFAs were determined in serum from control (Con), STZ-treated control (STZ-Con), and Tg1 transgenic (STZ-Tg) mice, as indicated in RESEARCH DESIGN AND METHODS. *$P < 0.05$ vs. Stz-Con.

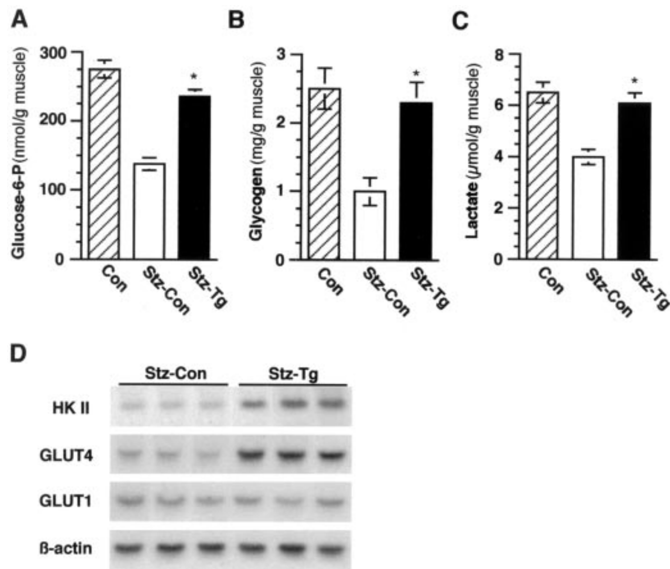

FIG. 3. Skeletal muscle glucose storage and utilization. Glucose-6-phosphate (Glucose-6-P) (*A*), glycogen (*B*), and lactate concentrations (*C*) were measured in perchloric extracts of skeletal muscle from healthy control (Con, ▨), STZ-treated control (STZ-Con, □) and Tg1 transgenic mice (STZ-Tg, ■), as described in RESEARCH DESIGN AND METHODS. Results are the mean ± SE of 10 mice in each group (*$P < 0.05$ vs. Stz-Con). *D*: Expression of hexokinase II (HK-II), glucose transporters GLUT4 and GLUT1, and β-actin in skeletal muscle of STZ-treated control (STZ-Con) and Tg1 transgenic mice (STZ-Tg) was determined by Northern blot analysis as described in RESEARCH DESIGN AND METHODS. A representative Northern blot is presented.

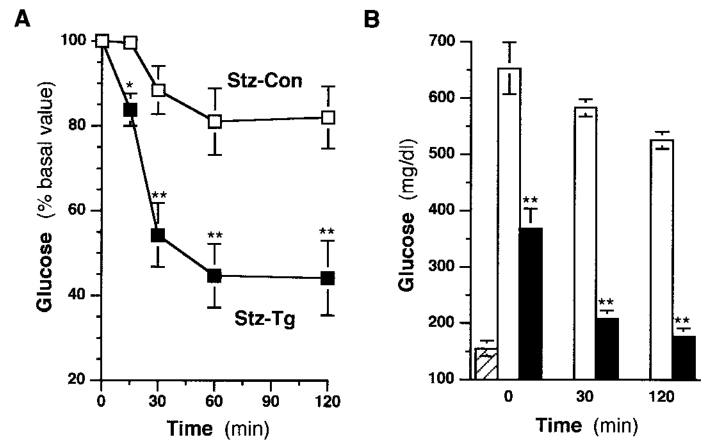

FIG. 4. Intraperitoneal insulin tolerance test. Forty days after STZ treatment awake control (□) and Tg1 transgenic (■) mice were intraperitoneally injected with 0.75 IU/kg body wt of insulin (Humulin regular; Eli Lilly), and at the indicated times, serum glucose was measured as indicated in RESEARCH DESIGN AND METHODS. Results are presented as percentage of serum glucose concentration before insulin injection (*A*) or as serum glucose concentration (*B*) (▨, healthy control; □, STZ-treated control (Stz-Con); ■, STZ-treated Tg1 transgenic mice (Stz-Tg)). Data are means ± SE of nine mice in each group (*$P < 0.05$ vs. Stz-Con; **$P < 0.01$ vs. Stz-Con).

shown). This indicates that low levels of insulin expression in skeletal muscle were unable to counteract diabetic alterations.

Local insulin production restored skeletal muscle and liver glucose metabolism in STZ-treated transgenic mice. Diabetic control mice showed a marked decrease (~60%) in the concentration of glucose-6-phosphate in skeletal muscle 1.5 months after STZ treatment. In contrast, STZ-treated Tg1 transgenic mice had similar levels to healthy controls (Fig. 3*A*). Skeletal muscle of STZ-treated Tg1 mice also showed higher glycogen (approximately twofold) and lactate (~50%) concentrations than those of STZ-treated controls and similar levels to those of healthy control mice (Fig. 3*B* and *C*). Glucose metabolism recovery in skeletal muscle of STZ-treated Tg1 mice paralleled increased expression of genes for HKII and GLUT4 compared with STZ-treated controls, while no changes were noted in GLUT1 gene expression (Fig. 3*D*). These results suggest that local production of insulin in skeletal muscle of STZ-treated transgenic mice led to increased GLUT4 content in the plasma membrane and to higher HKII activity and, thus, to normalization of muscle glucose metabolism.

Glucose phosphorylation by glucokinase (GK) regulates glucose utilization in the liver (26,27). An approximate 65% reduction in GK activity was noted in the liver of STZ-treated control mice, whereas STZ-treated Tg1 mice showed similar levels to healthy controls (Table 3). The reduction of GK activity in diabetic control mice led to a decrease (~60%) in the glucose-6-phosphate concentration. In contrast, STZ-treated Tg1 mice showed similar levels to healthy controls (Table 3). Furthermore, diabetic control mice showed reduced (~75%) glycogen content while STZ-treated Tg1 mice showed similar content to healthy controls (Table 3). These results suggest that the production of biologically active insulin by the skeletal muscle of STZ-treated Tg1 mice counteracted diabetic metabolic alterations, at least in part, by inducing skeletal muscle and liver glucose utilization.

Insulin treatment restored normoglycemia in fed STZ-treated transgenic mice. Forty days after administration of STZ, awake fed mice were intraperitoneally injected with 0.75 IU/kg body wt of soluble insulin (Humulin regular, Eli Lilly). Thirty minutes after insulin injection a marked decrease (~45%) in serum glucose concentration was noted in STZ-treated Tg1 mice, while only mild reduction (~10%) was detected in STZ-treated controls (Fig. 4*A*). By 60 min, STZ-treated transgenic mice reached normoglycemia and remained normoglycemic thereafter (Fig. 4*A* and *B*). In contrast, although STZ-treated control

TABLE 3
Hepatic glucose storage and utilization

Parameter	Con	STZ-Con	STZ-Tg
GK (mU/mg protein)	22 ± 3	8 ± 1	19 ± 2*
Glucose-6-P (nmol/g liver)	248 ± 11	103 ± 9	218 ± 12*
Glycogen (mg/g liver)	43 ± 4	11 ± 4	35 ± 3*

Data are the means ± SE of 10 mice in each group. GK activity and glucose 6-phosphate (Glucose-6-P) and glycogen concentrations were measured in liver extracts of healthy control (Con), STZ-treated control (STZ-Con), and Tg1 transgenic (STZ-Tg) mice as described in RESEARCH DESIGN AND METHODS. *$P < 0.05$ vs. Stz-Con.

mice showed a 20% reduction in serum glucose levels 60 min after insulin injection, they remained highly hyperglycemic (Fig. 4*A* and *B*). These results indicate that STZ-treated Tg1 mice were more sensitive to hypoglycemic effects of low doses of soluble insulin, which did not counteract hyperglycemia in STZ-treated control mice.

DISCUSSION

In this study, we show that expression of human insulin containing genetically engineered furin endoprotease cleavage sites led to the production of high levels of mature insulin in the skeletal muscle of transgenic mice. These results were consistent with those obtained in both hepatoma and muscle cells expressing the same mutated insulin gene (13,17), in which mature insulin was predominant in the culture medium. Furthermore, these transgenic mice did not show alterations of whole body glucose homeostasis and had normal lifespan and reproductive life. Because of the high insulin receptor levels in muscle fibers (40,41), insulin produced by skeletal muscle, acting in a paracrine/autocrine manner, may lead to increased disposal of glucose. However, fed transgenic mice were normoglycemic and normoinsulinemic, suggesting that skeletal muscle insulin production may have led to a compensatory decrease in pancreatic insulin secretion, thus maintaining normoglycemia and normoinsulinemia (42–46). In fasted conditions, because of the decrease in insulin secretion by β-cells and the constitutive production of insulin by skeletal muscle, Tg1 transgenic mice presented increased insulinemia and reduced glycemia compared with controls. These findings also suggest that, in addition to increased skeletal muscle glucose utilization, insulin produced by skeletal muscle may have contributed to higher glucose uptake by the liver and other insulin-sensitive tissues. This agrees with the increased glucose disposal observed after a glucose tolerance test in Tg1 transgenic mice. Similarly, transgenic mice with increased glucose uptake because their liver or skeletal muscle has been engineered to overexpress key genes in the regulation of glucose transport, such as GLUT4 (47–50) or GLUT1 (51), or glucose phosphorylation, such as glucokinase (52,53) or c-*myc* (33,54), show reduced blood glucose levels and improved glucose tolerance.

Forty-five days after STZ-treatment, fed control mice were highly hyperglycemic, while constitutive expression of insulin in skeletal muscle of Tg1 transgenic mice led to increased insulinemia and to marked reduction of hyperglycemia. Moreover, overnight-fasted STZ-treated Tg1 transgenic mice expressing insulin in skeletal muscle were normoglycemic and normoinsulinemic. The presence of normal serum insulin levels may have led to increased glucose disposal by skeletal muscle and all insulin-sensitive tissues, resulting in normalization of whole-body glucose metabolism. The lack of insulin in type 1 diabetes results in decreased GLUT4 levels and insulin-dependent glucose transport and utilization in skeletal muscle (28,29). However, insulin treatment normalizes glucose metabolism in this tissue. Similarly, skeletal muscle of STZ-treated Tg1 transgenic mice showed increased GLUT4 and HKII gene expression, as compared with STZ-treated controls, and normalization of glucose-6-phosphate, glycogen, and lactate concentrations, indicating that these mice had restored normal muscular metabolism in diabetic conditions. These results were consistent with an autocrine/paracrine role of insulin in skeletal muscle, which would lead to increased glucose uptake and utilization and contribute to the reduction of hyperglycemia. A key role of glucose transport and phosphorylation in regulating glucose metabolism of diabetic mice is also observed in transgenic mice overexpressing GLUT4 (55,56) or glucokinase (53) in skeletal muscle.

Furthermore, during diabetes, the liver does not take up glucose but releases glucose from gluconeogenesis to the blood (57). However, 45 days after STZ-treatment, the increased insulinemia of Tg1 transgenic mice resulted in increased GK activity that led to an increase in hepatic glucose metabolism, which may have contributed to the decrease in blood glucose levels and normalization of serum ketone body, triglycerides, and FFAs. Similarly, after STZ treatment, increased hepatic glucose utilization by overexpressing glucokinase (58) or c-*myc* (54) in the liver leads to normalization of hepatic glucose metabolism, reduction of diabetic hyperglycemia, and normalization of serum parameters.

This study also shows that fed STZ-treated Tg1 transgenic mice expressing insulin in skeletal muscle had a fast and strong hypoglycemic response to low doses of soluble, short-acting insulin, indicating that they were more sensitive to the hormone treatment. In contrast, STZ-treated control mice remained highly hyperglycemic. STZ diabetic nude mice develop insulin resistance and show only a slight hypoglycemic response to subcutaneous administration of 10 units short-acting insulin (59). Insulin resistance has also been observed in adults with childhood-onset type 1 diabetes (60–66) and may contribute to the high risk for cardiovascular disease in this population (60–62,66). Our findings in this transgenic model suggest that engineering skeletal muscle to produce basal levels of insulin during diabetes may be more effective in maintaining normoglycemia between meals than the administration of delayed-action insulin preparations. It may also reduce insulin resistance and macrovascular complications observed in type 1 diabetic patients. Thus, expression of insulin by skeletal muscle, in conjunction with short-acting insulin therapies, might maintain normal levels of blood glucose and delay secondary complications. However, in vivo gene transfer of insulin, using viral or nonviral vectors, to skeletal muscle of type 1 diabetic animal models must be performed before any gene therapy approach can be applied to humans.

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