Transgenic mice overexpressing phospho*enol*pyruvate carboxykinase develop non-insulin-dependent diabetes mellitus

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ABSTRACT   An increase in hepatic gluconeogenesis is believed to be an important factor responsible for the fasting hyperglycemia detected in patients with non-insulin-dependent diabetes mellitus (NIDDM). Phospho*enol*pyruvate carboxykinase (GTP) (PEPCK; EC 4.1.1.32) is a regulatory enzyme of gluconeogenesis. To study the role of the expression of PEPCK gene in the development of NIDDM, we have produced lines of transgenic mice expressing a PEPCK minigene under control of its own promoter. Transgenic mice were hyperglycemic and had higher serum insulin concentrations. In addition, alterations in liver glycogen content and muscle glucose transporter GLUT-4 gene expression were detected. The overexpression of the PEPCK gene led to an increase in glucose production from pyruvate in hepatocytes in primary culture. When intraperitoneal glucose tolerance tests were performed, blood glucose levels were higher than those detected in normal mice. This animal model shows that primary alterations in the rate of liver glucose production may induce insulin resistance and NIDDM.

Non-insulin-dependent diabetes mellitus (NIDDM), also known as type II diabetes, is by far the most common serious metabolic disorder, with a worldwide prevalence estimated to be between 1% and 6%, and is characterized by hyperglycemia and metabolic alterations (1–3). In its earliest stages, insulin deficiency is not detected, but a failure of the hormone to act in target tissues has been described (1–3). Despite considerable research effort, the causes of syndromes of insulin resistance are still not understood. Recent advances in the field have shown the high complexity of the problem. Several molecular defects have been associated with insulin resistance: e.g., genetic defects in the insulin receptor (4, 5), mutations in the gene for glucokinase (6, 7), or mutations in the glycogen synthase gene (8). However, these genetic alterations account for only a small number of cases of NIDDM. The primary causes of type II diabetes remain unknown in most cases.

A major factor contributing to fasting hyperglycemia in type II diabetes is excessive hepatic glucose production. Increased gluconeogenesis is responsible for the high blood glucose level in NIDDM after an overnight fast, which, in turn, causes an increased and sustained demand for insulin secretion (9–11). In the fasted state the rate of glucose utilization equals that of hepatic glucose output. In fasting patients with impaired glucose tolerance, the high plasma insulin cannot normalize glucose clearance (1). The cytosolic form of phospho*enol*pyruvate carboxykinase (GTP) (PEPCK; EC 4.1.1.32) is a regulatory enzyme of gluconeogenesis (12). PEPCK has no known allosteric modifiers and its activity is regulated by the transcription rate of its gene. Cyclic AMP and glucocorticoids increase liver PEPCK gene expression, whereas insulin has the opposite effect (13–15). Thus, this gene is highly sensitive to the main regulatory hormones of glucose homeostasis, glucagon and insulin.

We aimed to determine whether the overexpression of PEPCK led to a rise in the rate of gluconeogenesis; and, if so, to assess the contribution of the liver in the development of insulin resistance and NIDDM. To this end, we have produced transgenic mice carrying a rat PEPCK minigene. These mice showed that overexpression of a single enzyme, PEPCK, led to fasting hyperglycemia, decreased glycogen storage, and altered glucose tolerance test, features of NIDDM.

METHODS

Materials. DNA-modifying enzymes, the random-primed DNA labeling kit, nylon membranes, and dibutyryl cAMP were obtained from Boehringer Mannheim. [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. All media and sera were obtained from either Gibco Laboratories or Boehringer Mannheim. The other reagents used were of analytical grade. The rat PEPCK gene (pB7.0) and cDNA (pPCK10) were provided by Richard W. Hanson (Case Western Reserve University, Cleveland, OH). Rat glucose transporter GLUT-4 cDNA (pSM1-1-1) was provided by M. Birnbaum (Harvard University, Boston, MA).

Generation of Transgenic Mice. The 4.7-kb *Bam*HI–*Bam*HI fragment containing the PEPCK minigene was used to generate transgenic mice (see Fig. 1*A*). The general procedures for microinjection of the PEPCK minigene were as described (16). Fertilized mouse eggs were flushed from the oviducts of superovulated C57BL6/SJL mice 6–8 hr after ovulation. Male pronuclei of the fertilized eggs were injected with 2 pl of DNA solution ($\approx$2 ng/μl), and viable embryos were reimplanted in the oviducts of pseudopregnant mice. At 3 weeks of age, the animals were tested for the presence of the transgene by Southern blot analysis of tail DNA samples.

Mice were fed ad libitum with a standard diet (Panlab, Barcelona, Spain) and maintained under a light/dark cycle of 12 hr (lights on at 8:00 a.m.). When so stated, mice were starved for 24 hr. Blood samples were obtained between 10 and 11 a.m.

DNA and RNA Analysis. Transgenic mice were identified on Southern blots prepared with 10 μg of tail DNA digested with *Eco*RI. Blots were hybridized with a 1.7-kb *Sph* I–*Sph* I fragment of the rat PEPCK cDNA (17), radiolabeled with [α-^{32}P]dCTP by random oligonucleotide priming. Total RNA was isolated from liver or skeletal muscle by the guanidinium isothiocyanate method (18), and RNA samples (30 μg) were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde. Northern blots were hybridized to ^{32}P-labeled cDNAs. The PEPCK probe corresponded to the 1.7-kb *Sph* I–*Sph* I fragment of the rat PEPCK cDNA (17); the GLUT-4 probe corresponded to the 2.47-kb *Eco*RI–*Eco*RI fragment containing the entire rat GLUT-4 cDNA (19), and the β-actin probe corresponded to a 1.3-kb *Eco*RI–*Eco*RI fragment of the

Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; PEPCK, phospho*enol*pyruvate carboxykinase.
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rabbit cDNA. These probes were labeled with $[\alpha\text{-}^{32}P]dCTP$ by use of the random priming kit as described by the manufacturer (Boehringer Mannheim). Specific activity of the DNA probe thus labeled was $\approx 10^9$ cpm/μg of DNA. For autoradiography, blots were placed in contact with Kodak XAR-5 film. The β-actin signal was used to correct for loading inequalities.

Preparation and Incubation of Hepatocytes. Hepatocytes were isolated between 10 and 11 a.m. from fed mice as described (20). After removal of nonparenchymal cells and debris, hepatocytes were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 0.2% albumin and 10% fetal bovine serum. Cells were plated in 10 ml of this medium on collagen-coated dishes (5.5 × 10^6 cells in 10 ml per 100-mm-diameter dish) and maintained at 37°C under a 5% CO_2 atmosphere. After 4 hr the medium was removed and cells were washed three times in DMEM without glucose. Then 10 ml of DMEM without glucose and supplemented with 20 mM pyruvate was added to the cells, which were maintained in this medium up to 24 hr. Aliquots of 100 μl of medium were taken at various times and glucose production was determined.

Insulin and Metabolite Analysis. Insulin levels were measured by RIA (CIS, Biointernational, Gif-Sur-Yvette, France). Glucose was determined enzymatically (Glucoquant, Boehringer Mannheim, Germany). The concentration of hepatic glycogen was determined by the α-amyloglucosidase method (21) in perchloric acid extracts, which were adjusted to pH 5 with 5 M K_2CO_3.

Statistical Analysis. Any variability attributable to the experimental procedures was ruled out because in studies of individual RNA samples the correlation coefficient between increasing amounts of input RNA and signal intensity was 0.99 for all transcripts. To analyze the effects of perturbations

independently of variability in basal gene expression, we measured the densitometric readings for paired samples of nontreated control rats (always tested in the same Northern blot) after correction for loading differences with the actin signal. The data were summarized as the mean ± SEM. Statistical differences between data were analyzed with the Wilcoxon signed-rank test.

RESULTS

Generation of Transgenic Mice Expressing a PEPCK Minigene. To produce a PEPCK minigene, we used the 7.0-kb *Bam*HI fragment of the rat PEPCK gene (17), which contains the entire PEPCK coding sequence and 550 bp of the promoter/regulatory region. A 4.0-kb *Sph* I fragment of the PEPCK gene was removed (22) and replaced by the 1.7-kb *Sph* I fragment of the rat PEPCK cDNA (12, 22) (Fig. 1A). Regulatory elements in the PEPCK promoter which are responsive to cAMP, glucocorticoids, and insulin (23–25) are contained in the 550-bp segment. This region also confers tissue specificity, since it is able to direct the expression of chimeric genes mainly in the liver (26–28). In these animals the expression of the chimeric gene is regulated by diet in a similar manner to the endogenous PEPCK gene (26–28). Two founder mice expressing the PEPCK minigene (PP1 and PP2) were obtained when the minigene was microinjected into fertilized eggs (16). These transgenic animals carried about three intact copies of the PEPCK minigene when analyzed by Southern blot (Fig. 1B). To determine phenotypic consequences of PEPCK overexpression, both founder mice were bred to produce F_1 progeny. In the experiments described below, we used mice of F_1 and F_2 generations (2–4 months old) from the founder PP2, since they showed higher expression of the transgene. However, similar results were obtained
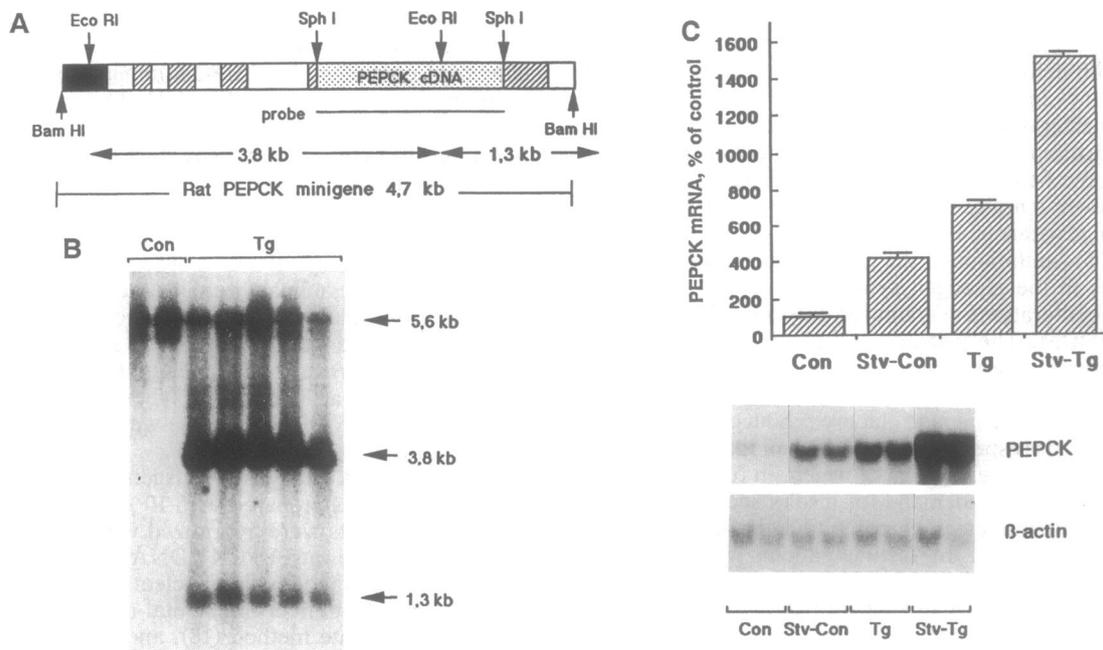

FIG. 1.   (A) PEPCK minigene used to create transgenic mice. A 7-kb fragment obtained from the pPCK-B7.0 plasmid (17) was used to generate the minigene. A 4.0-kb *Sph* I fragment of the PEPCK gene was removed (from exon 4 to exon 10) (17, 22) and replaced by the 1.7-kb *Sph* I fragment of the rat PEPCK cDNA (pPCK10) (17). The resulting minigene was 4.7 kb long. (B) Southern blot analysis of the presence of the PEPCK minigene DNA (10 μg) was digested with *Eco*RI restriction enzyme. Two restriction fragments (3.8 kb and 1.3 kb) were obtained that hybridized with the *Sph* I fragment of the PEPCK cDNA. The 1.3-kb fragment was produced by the restriction of *Eco*RI at the site of the PEPCK cDNA insertion and at the site of the 5' flanking region of a second molecule of the minigene, inserted in the genome in a head-to-tail manner. Control and transgenic mice showed a 5.6-kb fragment which hybridized with the cDNA probe, coming from the *Eco*RI restriction of the endogenous PEPCK gene. (C) Northern blot analysis of PEPCK mRNA levels. Mice were maintained under a light/dark cycle of 12 hr (lights on at 8:00 a.m.). Total RNA was extracted from liver of fed control (Con) and transgenic (Tg) mice (liver samples were obtained between 9 and 10 a.m.) and 24-hr-starved control (Stv-Con) and transgenic (Stv-Tg) mice. (*Upper*) Data are expressed as a percentage of the mRNA signal of control animals fed a standard diet (means and SEM of 10 mice in each group). (*Lower*) Representative Northern blot.

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with the line PP1. Littermates were used as controls for the transgenic animals. Transgenic mice did not show any sign of stress. They were healthy and grew normally, and no significant differences were detected in body weight when they were compared with control mice.

Previous studies performed in our laboratory showed that rat and mouse PEPCK mRNAs have identical size (data not shown). The concentration of the 2.8-kb PEPCK mRNA transcript in the liver was higher (7-fold) in transgenic than in control mice. This increase was noted in both fed and fasted conditions (Fig. 1C), indicating that the expression of the PEPCK minigene was regulated in a manner similar to that of the endogenous PEPCK gene.

Effects of PEPCK Overexpression in Cultured Hepatocytes from Transgenic Mice. To determine whether the increase in PEPCK expression led to an increase in gluconeogenesis, hepatocytes in primary culture from fed control and transgenic mice were incubated for up to 24 hr in DMEM without glucose and supplemented with pyruvate as a gluconeogenic precursor. A 2.5-fold induction of glucose production from pyruvate was noted in hepatocytes from transgenic mice (Fig. 2). This effect indicated that hepatocytes isolated from transgenic mice had a higher basal rate of gluconeogenesis than control animals.

Effects of PEPCK Overexpression in Glucose Homeostasis. Fed transgenic mice had 1.5-fold higher blood glucose concentration than control animals (Fig. 3A). Furthermore, transgenic mice showed a 3-fold increase in serum insulin (Fig. 3B). When mice expressing the PEPCK minigene were starved for 24 hr, the blood glucose levels were double those noted in control mice (Fig. 3A). An increase in glycemia during starvation is characteristic of NIDDM (1–3). In spite of the rise of serum insulin, transgenic mice showed a 60% reduction in liver glycogen, indicating insulin resistance (1–3) (Fig. 3C). Furthermore, in agreement with previous reports in other animal models of NIDDM (29, 30), transgenic mice also showed a reduction in muscle GLUT-4 mRNA concentration (Fig. 3D).

When the intraperitoneal glucose tolerance test was performed on mice starved for 24 hr, abnormally high levels of blood glucose were detected in transgenic mice. In contrast to control mice, the glucose levels reached in transgenic mice did not return to basal values 180 min after the intraperitoneal injection of glucose (Fig. 4). This impaired response to the glucose tolerance test suggested that transgenic mice expressing the PEPCK minigene developed diabetes.

DISCUSSION

In this report, we show that overexpression of the PEPCK gene leads to insulin resistance and altered glucose tolerance

FIG. 3. Serum glucose (A) and insulin (B) concentrations, liver glycogen content (C), and muscle mRNA encoding glucose transporter GLUT-4 (D) were determined as indicated in *Methods*. Results are means and SEM of 15 animals in each group. Con, fed control mice; Tg, fed transgenic mice; Stv-Con, 24-hr-starved control mice; Stv-Tg, 24-hr-starved transgenic mice.

test, which are features of NIDDM. Thus, these results indicate that an increase in PEPCK expression is sufficient to cause hepatic glucose overproduction in an animal without any other primary alteration. PEPCK catalyzes the first step of gluconeogenesis and the sole increase in this enzyme alone is sufficient to induce the metabolic pathway and finally to cause insulin resistance. There are other regulated steps (e.g., fructose-1,6-bisphosphatase, pyruvate kinase, transaminases) in addition to PEPCK which affect gluconeogenesis. However, since activation of PEPCK alone increases hepatic glucose output, it must have been a limiting step before activation. While defects in the enzymatic steps involved in glycolysis are now firmly linked to diabetes— e.g., glucokinase (maturity-onset diabetes of youth) (4, 7)—it is not established whether defects in the regulation of hepatic gluconeogenic enzymes might also cause diabetes.
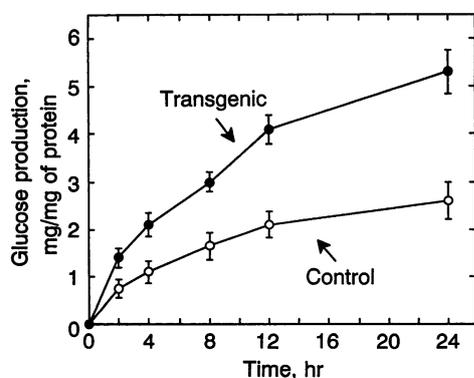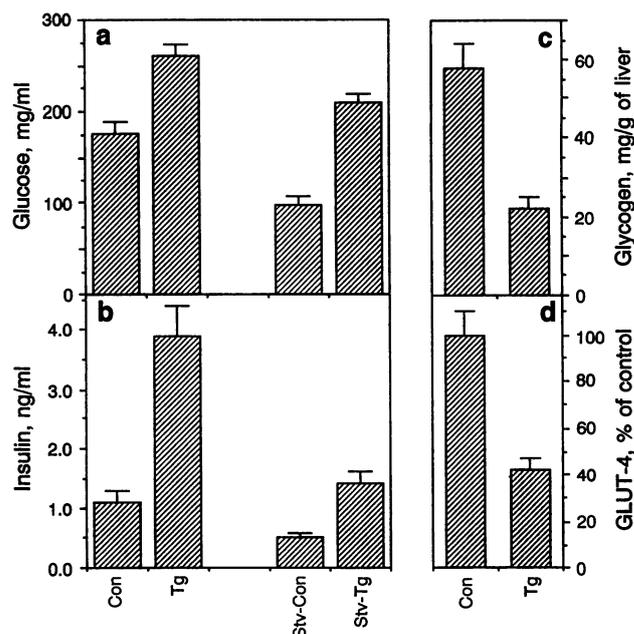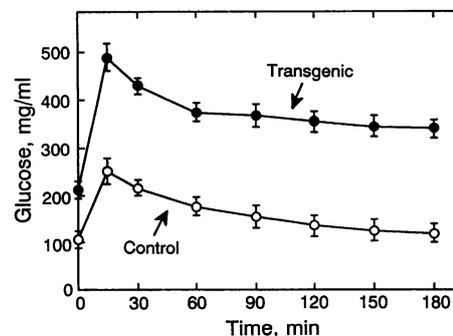
FIG. 2. Glucose production from pyruvate by hepatocytes in primary culture. Primary cultures of hepatocytes from fed control (o) and transgenic (●) mice were incubated in DMEM without glucose and with 20 mM pyruvate. At the indicated times an aliquot of the incubation medium was removed and glucose concentration was determined. Results are means ± SEM of three experiments.

FIG. 4. Intraperitoneal glucose tolerance test. Overnight-starved transgenic mice (●) had higher basal blood glucose levels than control mice (o). These mice were given an intraperitoneal injection of 1 mg of glucose per g of body weight. Blood samples were taken from the tail vein of the same animals at the times indicated. Glucose was determined in 30 µl of blood with a Reflotron (Boehringer Mannheim, Germany). Results are mean ± SEM of 10 transgenic and 10 control mice.

The primary cause(s) of NIDDM in most patients remains unknown. This study provides an animal model that suggests a general cause leading to insulin resistance. Transgenic mice overexpressing a PEPCK minigene in the liver share some of the main characteristics of NIDDM patients (1–3), and these results indicate that the liver might be involved in the development of this pathology. Therefore, if these findings can be extrapolated to humans, the sequence of events in the development of the disease in NIDDM patients would be as follows: (*i*) continued high blood glucose levels, which could stem from a variety of sources—e.g., as a consequence of a primary increase in PEPCK activity; (*ii*) to prevent hyperglycemia, β cells secrete more insulin; and (*iii*) diabetes mellitus eventually develops in individuals whose β cells do not meet the sustained increased demand for insulin.

Transgenic mice overexpressing PEPCK may provide further insight into the mechanism(s) by which primary hyperglycemia (in an otherwise healthy animal) leads to insulin resistance and diabetes. Furthermore, the transgenic model presented in this study might also be used in the development of new therapies, other than insulin treatment, for diabetes. It has been observed that the expression of PEPCK is increased in patients with type II diabetes, which leads to abnormally high hepatic glucose production (9–11). Therefore, specific inhibitors of either PEPCK gene expression or PEPCK activity might decrease hepatic glucose overproduction and substantially decrease glycemia in these patients.

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