Phosphoenolpyruvate Carboxykinase Overexpression Selectively Attenuates Insulin Signaling and Hepatic Insulin Sensitivity in Transgenic Mice*

Yang Sun‡, Sha Liu‡, Sandra Ferguson‡, LiQin Wang‡, Patrick Klepcyk‡, Jeung S. Yun‡, and Jacob E. Friedman‡‡

From the ‡Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4935 and the §Edison Animal Biotechnology Center, Ohio University, Athens, Ohio 45107

The ability of insulin to suppress gluconeogenesis in type II diabetes mellitus is impaired; however, the cellular mechanisms for this insulin resistance remain poorly understood. To address this question, we generated transgenic (TG) mice overexpressing the phosphoenolpyruvate carboxykinase (PEPCK) gene under control of its own promoter. TG mice had increased basal hepatic glucose production (HGP), but normal levels of plasma free fatty acids (FFAs) and whole-body glucose disposal during a hyperinsulinemic-euglycemic clamp compared with wild-type controls. The steady-state levels of PEPCK and glucose-6-phosphatase mRNAs were elevated in livers of TG mice and were resistant to down-regulation by insulin. Conversely, GLUT2 and glucokinase mRNA levels were appropriately regulated by insulin, suggesting that insulin resistance is selective to gluconeogenic gene expression. Insulin-stimulated phosphorylation of the insulin receptor, insulin receptor substrate (IRS)-1, and associated phosphatidylinositol 3-kinase 3-kinase were normal in TG mice, whereas IRS-2 protein and phosphorylation were down-regulated compared with control mice. These results establish that a modest (2-fold) increase in PEPCK gene expression in vivo is sufficient to increase HGP without affecting FFA concentrations. Furthermore, these results demonstrate that PEPCK overexpression results in a metabolic pattern that increases glucose-6-phosphatase mRNA and results in a selective decrease in IRS-2 protein, decreased phosphatidylinositol 3-kinase activity, and reduced ability of insulin to suppress gluconeogenic gene expression. However, acute suppression of HGP and glycolytic gene expression remained intact, suggesting that FFA and/or IRS-1 signaling, in addition to reduced IRS-2, plays an important role in downstream insulin signal transduction pathways involved in control of gluconeogenesis and progression to type II diabetes mellitus.

Type II diabetes mellitus is a complex metabolic disease with an environmental and genetic component. Hyperglycemia develops for reasons that are not completely understood; however, a prominent defect is the inability of insulin to inhibit hepatic glucose production (HGP). Increased gluconeogenesis is believed to be the major cause of increased HGP and fasting hyperglycemia in type II diabetes mellitus patients (1). Gluconeogenesis is a highly regulated process, catalyzed by several enzymes subject to regulation by insulin. The first site of regulation, the conversion of oxaloacetate to phosphoenolpyruvate, is catalyzed by the enzyme phosphoenolpyruvate carboxykinase (PEPCK). PEPCK is considered a key rate-controlling enzyme in the pathway of gluconeogenesis from pyruvate, lactate, and alanine (2). Normally, insulin rapidly and substantially inhibits PEPCK gene transcription and gluconeogenesis in liver and in rat hepatoma cells. However, in several animal models of obesity and type II diabetes, gluconeogenesis and PEPCK mRNA are increased by 2–3-fold over non-diabetic animals, despite circulating insulin levels that may be 4–10-fold greater than those in non-diabetic controls (3–5). These results suggest that hyperglycemia in these animal models might be secondary to insulin signaling defects resulting in lack of suppression of PEPCK gene transcription.

In addition to regulating glycolytic and gluconeogenic enzymes in the liver, insulin controls gluconeogenesis by regulating the concentration of plasma free fatty acids (FFAs). The liver has a high capacity to remove FFAs from the portal circulation. The main function of increased FFAs during fasting is to generate the ATP necessary to shift the equilibrium of the tricarboxylic acid cycle toward the net synthesis of glucose from pyruvate. Although clinical studies support the stimulatory effects of FFAs, particularly derived from visceral lipolysis, on HGP and insulin resistance (6–9), the major intrahepatic mechanism(s) underlying the failure of insulin to suppress gluconeogenesis in type II diabetes remains elusive.

Insulin also decreases transcription of the gluconeogenic genes encoding fructose-1,6-bisphosphatase and glucose-6-phosphatase while increasing the expression of the glycolytic enzymes glucokinase and pyruvate kinase. Targeted overexpression or gene knockouts of PEPCK, glucose-6-phosphatase, glucokinase, and GLUT2 (glucose transporter-2) demonstrate that altered enzyme expression in the liver can play a major regulatory role in hepatic metabolism and whole-animal glucose homeostasis (10–14). However, a detailed study of the role of extracellular FFAs and the ability of insulin to suppress HGP in vivo has not been carried out in most of these trans-

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†To whom correspondence should be addressed: Dept. of Pediatrics, Biochemistry, and Molecular Genetics, University of Colorado Health Sciences Center, Mail Stop B195, 4200 East Ninth Ave., Denver, CO 80262. Tel.: 303-315-5130; Fax: 303-315-3851; E-mail: jed.friedman@UCHSC.edu.

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1 The abbreviations used are: HGP, hepatic glucose production; PEPCK, phosphoenolpyruvate carboxykinase; FFA, free fatty acid; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; G6Pase, glucose-6-phosphatase; TG, transgenic; WT, wild-type; IR, insulin receptor; MOPS, 4-morpholinepropanesulfonic acid.
genetic models. It is important to recognize that gluconeogenesis is a coordinated pathway and that sites other than genes encoding gluconeogenesis may also contribute to hepatic insulin resistance to suppress HGP. For example, recent studies have suggested that hepatic insulin resistance might be associated with a loss of insulin receptor substrate (IRS)-2-associated phosphatidylinositol 3-kinase (PI3K) activity (15). A crucial feature of this hypothesis is that hyperinsulinemia normally suppresses IRS-2 transcription, and when IRS-2 is down-regulated by chronic hyperinsulinemia or gene knockout, there is a loss of insulin regulation of HGP (16–18). This creates a paradox in that down-regulation of IRS-2 expression by insulin must be intact to suppress its mRNA, whereas PEPCK is inappropriately elevated despite hyperinsulinemia.

A previous study by Valera et al. (10) demonstrated that mice overexpressing a PEPCK minigene driven by the PEPCK promoter develop fasting hyperglycemia, hyperinsulinemia, and impaired glucose tolerance. Although these results suggest that solely increasing PEPCK activity in the liver can result in hyperinsulinemia, β-cell failure, and diabetes, it is unclear whether this is due to a failure of insulin to suppress insulin signal transduction in the liver, a defect in insulin suppression of lipolysis, or a secondary effect of hyperglycemia on skeletal muscle glucose uptake. In mice with a liver-specific insulin receptor knockout, a loss of insulin signaling was associated with a 5-fold increase in IRS-2, elevated PEPCK, and mild fasting hyperglycemia (19). Surprisingly, basal HGP was actually decreased in the liver-specific insulin receptor knockout mice compared with controls, due perhaps to 20-fold higher insulin levels that significantly suppressed serum triglycerides and FFAs. Thus, deregulation of some systemic components of the gluconeogenic pathway may play an important role in increased HGP of diabetes.

The purpose of this study was to determine the mechanism(s) whereby overexpression of PEPCK impairs the ability of insulin to suppress HGP. In particular, we considered the role of regulation of hepatic genes by insulin and the signal transduction pathways that mediate these responses. We show that a modest (2-fold) increase in PEPCK gene expression is sufficient to increase basal HGP and to trigger impaired glucose tolerance, hallmarks of type II diabetes mellitus, without affecting plasma FFA concentrations. Our results also establish that PEPCK overexpression results in a metabolic pattern that increases glucose-6-phosphatase (G6Pase) mRNA expression in the livers of transgenic (TG) mice and that results in a selective decrease in IRS-2 protein, decreased PI3K activity, and reduced ability of insulin to suppress gluconeogenic gene expression. However, the acute suppressive effects of insulin on HGP, FFAs, and other genes appear to be intact in TG mice, suggesting that a select modification of metabolic pathways occurs in response to increased gluconeogenic gene expression.

EXPERIMENTAL PROCEDURES

**Experimental Animals**—The PEPCK promoter has been used by numerous investigators to drive overexpression of foreign genes in the livers of transgenic mice. Our constructs are similar to previous PEPCK gene constructs used routinely at the Ohio University Edison Biotechnology Institute and in other laboratories to generate transgenic mice (10, 20–22). The minigene construct used for microinjection contains 6.1 kb of the rat PEPCK gene, including 460 bp of 5′-flanking sequence (23). The PEPCK gene was coupled to a Smal-EcoRI polyadenylation signal from the bovine growth hormone gene (see Fig. 1A) (24). The bovine growth hormone polyadenylation signal was chosen because it is known to increase mRNA stability in transgenic mice (20). The methods for production and characterization of transgenic mice have been described in detail previously (20, 22). Founder transgenic mice were bred with non-transgenic B6SJL mice to generate male and female heterozygous mice. Heterozygous males and females from each line were bred homozygously and propagated up to four generations. The presence of the transgene was determined by PCR analysis of genomic DNA obtained from tail biopsy and confirmed by Southern blotting. Age-matched 10–14-week-old wild-type (WT) littermates were used as controls. WT and TG mice were housed in microisolator cages and maintained on a fixed 12-h light/dark cycle. Animals had free access to water and standard AIN-76A diet (Harlan Teklad, Madison, WI) ad libitum. The normal mouse chow diet used in these studies was Teklad F6 8664, containing 24% protein, 6% fat, 4.5% crude fiber, and the remainder carbohydrate.

**Glucose Challenge Test**—Studies were performed as described previously (25). For several days before each study, mice were accustomed to the experimental environment. All TG mice were given an intraperitoneal injection of 36 mg/kg body weight for glucose tolerance testing. Blood (5–10 μl) was sampled from the tail vein and assayed for glucose and insulin at the times shown, without re-clipping of the tail.

**Hyperinsulinemic-Euglycemic Clamp Studies**—Mice underwent a two-step hyperinsulinemic-euglycemic clamp using n-[3-3H]glucose to measure the rate of appearance of glucose, HGP, and total body glucose utilization according to Ren et al. (26) as reported previously by us (25, 27). Three days after jugular catheter placement, a bolus followed by constant n-[3-3H]glucose infusion was maintained throughout the study. The first step was initiated with an insulin infusion (regular human; Lilly) at 5 milliunits/kg/min, and a variable unlabeled glucose (25% dextrose) infusion was used to maintain glucose levels at 100 ± 10 mg/dl. The second step of the study was initiated by increasing the insulin infusion rate to 20 milliunits/kg/min. Similar procedures were followed as outlined for the first step. After completing the second step, the animals were killed by overdose of anesthetic (ketamine), and tissues were removed, frozen immediately in liquid nitrogen, and stored at −70 C.

**Calculations**—The rate of glucose appearance (Ra; mg/kg/min), which equals the rate of total body glucose utilization (Ra) during steady state, was calculated as the ratio of the rate of infusion of [3-3H]glucose (dpm/min) and the steady-state plasma [3H]glucose specific radioactivity (dpm/μmol). The rate of HGP (mg/kg/min) during the insulin clamp was calculated as the difference between the tracer-derived Ra and the rate of the unlabeled glucose infusion.

**Insulin Receptor, IRS, and PI3K and Their Phosphorylation**—To observe the effects of PEPCK overexpression on insulin signal transduction, insulin (1 milliunit/kg of body weight) was administered to anesthetized mice via the portal vein as outlined previously (28). Liver samples were removed within 2 min and compared with livers obtained from anesthetized mice after an overnight fast. Samples were stored at −80 °C until analysis. For insulin receptor (IR)-β, IRS-1, IRS-2, and phosphorylated IRS-2 analyses, liver samples were homogenized in 10 volumes of solubilization buffer and centrifuged for 1 h, and the supernatant was used for Western blot analysis using methods described previously (25).

The level of tyrosine phosphorylation was determined after immunoprecipitation with anti-phosphotyrosine antibodies, followed by Western blot analysis using SDS-PAGE and immunoblotting with anti-IR, anti-IRS-1, anti-IRS-2, and anti-phos. Antibodies. IRS-1 and IRS-2 associated PI3K activity was measured in extracts after immunoprecipitation overnight at 4 °C (400 μg of muscle protein/μg of antibody), followed by overnight incubation with protein A-Sepharose. The immunoprecipitation complex was spun at 14,000 × g for 10 min, followed by washing three times with isotonic phosphate-buffered saline. The PI3K reaction contained 10 μl of ATP mixture with 100 μmol MgCl2, 10 mm Tris (pH 7.5), 0.55 mm ATP, and 1 μCi/ml [γ-32P]ATP added for 10 min at room temperature. The reaction was stopped with 20 μl of 8 N HCl, and 5 min later, 160 μl of CHCl3/MeOH (1:1). The phases were separated by centrifugation, and the lower organic phase was removed, lyophilized to dryness, and resuspended in 15 μl of ethanol. The lipids were resolved on a layered TLC plate precoated with 1% potassium oxalate, dried, and visualized by autoradiography. The images were scanned using a Kodak Digital Phosphomager, and results (in duplicate) are expressed as % stimulation over basal (arbitrary units) relative to the WT control.

**RNA Extraction and Northern Blot Analysis**—Total RNA was extracted from the livers of fasted control and insulin-clamped animals using the guanidine thiocyanate procedure as described (25). 20 μg of RNA/sample was loaded onto a 0.8% formaldehyde-containing 1% agarose gel.
Fig. 1A. PEPCK gene construct used in the generation of transgenic mice. The entire PEPCK gene including 460 bp from the 5'-upstream regulatory region was ligated to a bovine growth hormone (bGH) polyadenylation sequence. The black boxes represent the exon and intron boundaries. Representative Northern blot analysis of livers from three separate transgenic lines demonstrated that the total amount of PEPCK mRNA normalized to 28 S RNA was increased by 2-fold in TG mice compared with WT mice at 10 weeks of age. B and C, glucose and insulin levels, respectively, during a tolerance test in WT (■) and PEPCK-TG (○) mice. Glucose tolerance tests were carried out in mice fasted for 6 h and injected intraperitoneally with 2 g of glucose/kg of body weight. Plasma glucose and insulin in samples obtained from tail veins were measured as described under “Experimental Procedures.” Results are means ± S.E. of 6–12 animals/group. *, significantly greater compared with WT controls (p < 0.05).

TABLE I

|              | WT   | TG   |
|--------------|------|------|
| Body weight (g) | 24.8 ± 1.4 | 24.4 ± 1.7 |
| Glucose (mg/dl)   | 100 ± 9 | 108 ± 5  |
| Insulin (ng/ml)    | 1.8 ± 0.2 | 4.2 ± 0.2  |
| FFAs (mM)          | 0.68 ± 0.08 | 0.63 ± 0.05 |
| Lactate (mg/dl)    | 26.3 ± 3.3 | 21.0 ± 0.95  |
| Corticosterone (ng/ml) | 28.2 ± 10.7 | 24.1 ± 14.6  |
| Plasma triglycerides (mg/dl) | 67.7 ± 10.1 | 74.3 ± 13.1  |
| Hepatic triglycerides (mg/liver) | 35.3 ± 7.4 | 34.5 ± 6.6  |

* Significantly different (p < 0.05).

Effect of PEPCK Overexpression on Glucose Tolerance and Hyperinsulinemic-Euglycemic Clamp Studies—Increased fasting insulin levels in TG mice suggested that the mice were insulin-resistant. Glucose tolerance tests were therefore carried out in 6-h fasted mice (Fig. 1, B and C). The levels of glucose were significantly elevated in TG mice throughout the glucose tolerance test (p < 0.01), whereas insulin levels were increased at time 0 and at 30 min, suggestive of glucose intolerance. To further delineate the source of the insulin resistance, hyperinsulinemic-euglycemic clamp studies were performed using 0.1-mU/kg/min insulin to measure HGP and total body glucose utilization under steady-state conditions (Fig. 2). Under basal conditions prior to the insulin clamp, HGP was 35% greater in TG mice (p < 0.05). However, during low-dose insulin infusion at the same plasma insulin concentration, HGP was suppressed by similar absolute amounts in WT and TG transgene had no significant effect on levels of PEPCK mRNA expressed in the kidney compared with WT mice (data not shown). Table I provides a summary of metabolic parameters in WT and TG mice assayed under fasting conditions. Fasting plasma glucose levels were unchanged; however, TG mice had a 3-fold increase in fasting insulin levels (p < 0.05). Among the factors that promote gluconeogenesis, plasma FFA and corticosterone values were not different between groups; however, the gluconeogenic precursor lactate was 20% lower in TG mice (p < 0.05). Hepatic and circulating triglyceride levels were normal in TG mice.

RESULTS

Metabolic Profile of Adult PEPCK-TG Mice—To determine the potential role of PEPCK overexpression in hepatic glucose output and gene regulation in vivo, we generated transgenic mice overexpressing the PEPCK gene under control of its own promoter. Transgene expression in this model is directed by 460 bp of the 5’-regulatory region of the PEPCK promoter and has been found to contain all of the necessary elements for developmental, hormonal (including cAMP, insulin, and glucocorticoids), and dietary control in the liver in a manner similar to the endogenous PEPCK gene promoter (3, 22). Three founder transgenic mouse lines were bred to homozygosity. Growth curves and body weights in TG and WT mice were similar throughout postnatal development on a normal chow diet (data not shown). Northern blot analysis of their livers at 10 weeks of age showed a 2-fold increase (p < 0.05) in PEPCK mRNA in TG mice compared with WT controls (Fig. 1A). The
the jugular vein, and glucose turnover was measured with [3-3H]glucose in the basal state (time 0), followed by a two-step hyperinsulinemic-euglycemic infusion to determine insulin sensitivity and suppression of HGP. Insulin was infused at a rate of 5 milliunits/kg/min for up to 90 min, followed by 20 milliunits/kg/min for a total of 3-3.5 h, depending on the ability to achieve a steady-state glucose concentration. Blood glucose was determined every 10 min, and the rate of unlabeled glucose infusion (20% glucose) was adjusted to maintain euglycemia. Steady-state specific glucose radioactivity and blood glucose concentrations were achieved during the last 30 min of each step of the clamp and used to calculate glucose disposal rates and rates of HGP.

Data are means ± S.E. of 8-10 mice/group. *, p < 0.05 versus WT mice.

### Effects of Insulin on FFAs, Leptin, and Hepatic Gene Expression in PEPCK-TG Mice

Mice were chronically catheterized and glucose-maintained between 90 and 110 mg/dl during the insulin clamp by determining glucose every 10 min and adjusting the rate of unlabeled glucose infusion (20% glucose) to maintain euglycemia. Insulin was infused at a rate of 5 milliunits/kg/min for up to 90 min, followed by 20 milliunits/kg/min for a total of up 4 h, depending on the ability to achieve a steady-state glucose concentration. Values are means ± S.E. (n = 8-10 mice in each group).

**TABLE II**

|                | Pre     | Post    |
|----------------|---------|---------|
| **FFAs (mM)** |         |         |
| WT             | 1.01 ± 0.06 | 0.13 ± 0.07 |
| TG             | 0.88 ± 0.08 | 0.11 ± 0.10 |
| **Leptin (ng/ml)** |       |         |
| WT             | 1.2 ± 0.3 | 1.5 ± 0.2 |
| TG             | 1.1 ± 0.2 | 1.3 ± 0.1 |

### Results

#### Plasma concentrations of free fatty acids and leptin at base line and following hyperinsulinemic clamp studies

Mice were chronically catheterized and glucose-maintained between 90 and 110 mg/dl during the insulin clamp by determining glucose every 10 min and adjusting the rate of unlabeled glucose infusion (20% glucose) to maintain euglycemia. Insulin was infused at a rate of 5 milliunits/kg/min for up to 90 min, followed by 20 milliunits/kg/min for a total of up 4 h, depending on the ability to achieve a steady-state glucose concentration. Values are means ± S.E. (n = 8-10 mice in each group).

**FIG. 2.** Rates of HGP and glucose disposal (Rd) during the basal period and during a hyperinsulinemic-euglycemic clamp in WT and PEPCK-TG mice. Mice were chronically catheterized via the jugular vein, and glucose turnover was measured with [3-3H]glucose. Data are means ± S.E. of 8-10 mice/group. *, p < 0.05 versus WT mice.

**FIG. 3.** mRNA expression levels of PEPCK, G6Pase, glucokinase, and GLUT2 in the livers of overnight fasted mice and in response to the hyperinsulinemic-euglycemic clamp. Total RNA was isolated from the livers of mice under overnight fasting conditions (Control) or following insulin-clamped conditions (+ Insulin). The levels of PEPCK, G6Pase, glucokinase, GLUT2, and ribosomal mRNAs were detected as described under “Experimental Procedures.” The relative levels of mRNA were determined by densitometry and expressed as a percentage of mRNA hybridization (arbitrary units) from wild-type control livers detected on the same Northern blot. All values were corrected for ribosomal mRNA to account for loading differences. Results are means ± S.E. of four to six animals/group. *, p < 0.05, TG versus WT mice (no insulin); #, p < 0.05, insulin-stimulated versus controls (no insulin).

To determine whether insulin regulation of hepatic expression of key gluconeogenic and glycolytic genes was intact, Northern blot analysis was performed in livers from fasted animals and following the hyperinsulinemic-euglycemic clamp (Fig. 3). Prior to the hyperinsulinemic clamp, the basal levels of PEPCK, G6Pase, and glucokinase were significantly increased by 2-fold in TG mice compared with WT mice (p < 0.05). In WT mice, insulin suppressed the levels of PEPCK, G6Pase, and GLUT2 mRNAs markedly by ~50-85% (p < 0.05); however, hyperinsulinemia had no significant effect on reducing PEPCK and G6Pase mRNA levels in TG mice. Similarly, insulin increased glucokinase mRNA levels by 2-fold in WT mice (p < 0.05), but glucokinase was not increased further in TG mice. The basal levels of GLUT2 mRNA were similar between TG and WT mice and were suppressed to the same extent (75%; p < 0.05) following insulin infusion.

**Decreased IRS-2-associated P38 Kinase Activity in Livers from TG Mice**—Given the reduced ability of insulin to suppress PEPCK and G6Pase mRNA expression in TG mice, we next determined whether this was associated with impaired insulin signal transduction in the livers of TG mice. Mice were injected with insulin; after 2 min, the livers were removed, and proteins were analyzed by Western blotting using anti-phosphotyrosine antibodies, followed by immunoblotting with IR, IRS-1, IRS-2, and the p85α subunit of PI3K (Fig. 4). Western blot analysis revealed no significant differences in the amount of IR, IRS-1, and IRS-2, and the p85α subunit of PI3K. In contrast, the amount of the IRS-2 was decreased by ~2-fold in TG mice versus controls (p < 0.05). After insulin injection, tyrosine phosphorylation of IRS-1 and IRS-2 was not significantly different between groups. Likewise, PI3K activity associated with phosphorylated IRS-1 was not affected in TG mice. However, consistent with reduced IRS-2 expression, tyrosine phosphorylation of IRS-2 was reduced by 55% (p < 0.05), and the levels of IRS-2-associated P38K activity were reduced by 43% in TG mice. IRS-2 association with the p85α subunit of P3K was also reduced in TG mice (p < 0.05) (data not shown). These results indicate that the loss
of insulin signaling in the liver is specific to IRS-2 and includes decreases in IRS-2-associated PI3K activity.

**DISCUSSION**

The results of this study show that a modest (2-fold) increase in PEPCK gene expression is sufficient to increase HGP, a hallmark of type II diabetes mellitus, independent of a change in plasma FFA concentration. Although not sufficient to cause fasting hyperglycemia due to compensatory hyperinsulinemia, overexpression of the enzyme clearly caused a condition of glucose intolerance (Fig. 1). Doubling the levels of expression of the cytosolic form of PEPCK increased basal HGP by 33%, and plasma lactate was lower by 20%. This suggests that although PEPCK-C is a dominant control point, there is very likely multienzyme and/or substrate regulation of the gluconeogenic pathway. Elevated PEPCK expression increased the rate of HGP without affecting peripheral insulin resistance as evidenced by normal peripheral glucose disposal during the hyperinsulinemic-euglycemic clamp (Fig. 2). These results are novel and suggest that the primary mechanism underlying impaired glucose tolerance in TG mice is due to a defect in insulin action in the liver, rather than a change in adipose tissue or skeletal muscle. This model differs significantly from previous transgenic mice in which a 7-fold increase in PEPCK expression was primarily restricted to the liver, and the mice had a normal growth pattern and adipose tissue mass. Some investigators have speculated that increased hepatic glyceroneogenesis could lead to increased triglyceride storage, independent of plasma FFA release from adipose tissue (33, 34). However, the physiological role of glyceroneogenesis from pyruvate in the regulation of hepatic triglyceride synthesis remains to be determined. In this study, increased PEPCK expression had a much greater effect on glucose production than on triglyceride formation. One reason for lack of an increase in hepatic triglycerides could be due to the fact that de novo lipogenesis in mice makes up a very small fraction of the total triglyceride pool in the liver. Other models in which the triglyceride content of the liver is elevated have increased white adipose tissue mass, along with increased adipose-derived FFAs, which may be necessary for triglyceride synthesis (35).

Our results also establish that PEPCK overexpression in liver results in a metabolic pattern that increases G6Pase mRNA expression in the livers of TG mice. Insulin normally decreases the activity and mRNA levels of the G6Pase catalytic subunit (36). Our results demonstrated that both G6Pase and PEPCK mRNAs were resistant to suppression during the hyperinsulinemic-euglycemic clamp, suggesting that the livers were highly insulin-resistant. Studies in vivo (37) and in vitro in isolated hepatocytes (38) have shown that a small increase in glucose concentration as low as 11 mM can induce G6Pase gene transcription up to 20-fold. Furthermore, when glucose is metabolized via glucokinase overexpression in hepatoma cells, the inhibitory effect of insulin is blunted, and the stimulatory effect of glucose on G6Pase gene expression becomes dominant (38). Although glucose utilization was not measured directly in this study, the level of glucokinase mRNA was elevated 2-fold in the livers of TG mice. These findings suggest that hepatic insulin resistance and/or elevated glucose levels in the hepatocyte may have caused an increase in G6Pase expression and contributed to greater HGP in TG mice as a pathological consequence. Although our conclusion is based primarily on measurements of the levels of mRNAs in the livers of the mice, overexpression of G6Pase is known to stimulate HGP (14, 39) and is consistent with the metabolic variations that occur in response to PEPCK

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**FIG. 4.** Representative immunoblots and quantification of insulin signaling proteins in livers from WT and PEPCK-TG mice *in vivo*. Mice were fasted overnight and anesthetized, and insulin was injected into the portal vein (1 milliunits/kg of body weight). Samples were removed within 2 min after insulin treatment. Proteins from fasted and insulin-treated animals were analyzed for phosphorylation by immunoprecipitation (IP) with an anti-phosphotyrosine antibody, followed by immunoblotting. IRS-1- and IRS-2-associated PI3K (PI 3-kinase) activity was determined in protein extracts after immunoprecipitation as described under “Experimental Procedures.” The results are expressed as arbitrary units above basal levels prior to insulin injection. Data are means ± S.E. of four to six samples/group. *, p < 0.05, WT versus TG mice.
overexpression in the liver, including a defect in insulin signaling as shown here.

Another important finding of this study is that PEPCK overexpression in the liver results in a selective decrease in IRS-2 protein, decreased P13K activity, and reduced ability of insulin to suppress gluconeogenic mRNA expression. Most (40–43), but not all (44), studies suggest that insulin signaling via PI3K to Akt is critical in suppressing the activity of the PEPCK and G6Pase promoters. More recently, it was found that Akt2 knockout mice have impaired suppression of HGP accompanied by hyperinsulinemia (45), although the later phenotype could be due to a compensatory response to lack of Akt2 in other tissues. The chronic increase in gluconeogenic mRNAs and glucokinase in PEPCK-TG mice is similar to lipodystrophic and ob/ob mice, which have a substantial loss of IRS-2 expression in the liver (17). Goldstein and co-workers (46) suggested that increased PEPCK mRNA in these mice was secondary to an effect of hyperinsulinemia on decreasing transcription of the IRS-2 gene necessary for regulating PI3K activity and phospho-Akt. Our results suggest that increased PEPCK expression can initiate a gluconeogenic program that results in hyperinsulinemia, down-regulated IRS-2 expression, and dysregulation of gene transcription, which then accelerates insulin resistance to suppress hepatic gene expression.

An unexpected finding is that despite reduced IRS-2-associated PI3K activity, insulin has a similar acute effect on suppressing HGP in TG and WT mice (~9 mg/kg/min). Although the basal rates of HGP were higher in TG mice, these findings suggest that the acute versus chronic effects of insulin on suppressing HGP may be mediated by different mechanisms. Interestingly, the insulin receptor and IRS-1-associated PI3K activity were totally normal in TG mice. These results bring into question the notion that IRS-2 controls all of the signals that regulate the effect of insulin on HGP. It should be noted that although IRS-2 knockout mice are hyperglycemic and insulin-resistant, heterozygous IRS-2-/+ mice, with IRS-2 levels similar to those found in this study, show normal suppression of HGP by insulin (15). Thus, in this scenario, we speculate that there is a selective form of insulin resistance in the livers of transgenic mice. Although many of the metabolic abnormalities in PEPCK-overexpressing mice can be related to defects in insulin-regulated pathways that control gene expression, the ability of insulin to acutely inhibit post-transcriptional regulatory enzymes may have strongly influenced hepatic fluxes. In addition, the normal suppression of plasma FFAs by insulin demonstrated in TG mice may play an important role in the moment-to-moment suppression of liver glucose output by mechanisms other than gluconeogenic regulation. It is therefore tempting to speculate that the acute suppression of plasma FFAs and HGP, despite the inappropriate mRNA expression profile, may have prevented the TG mice from converting from impaired glucose tolerance to overt fasting hyperglycemia.

The results also provide direct evidence that increased PEPCK gene expression induces a gluconeogenic program that results in reduced insulin signaling in the liver, particularly IRS-2, which may play a role in insulin resistance to suppress gluconeogenic gene expression. In FAO hepatoma cells, hyperinsulinemia can trigger IRS-2 degradation, but fails to promote IRS-1 degradation (47). Thus, degradation of IRS-1 and IRS-2 may be mediated by different mechanisms under these circumstances. The fact that a select pool of PI3K is required for gluconeogenic gene regulation by insulin may reflect differences in downstream substrate specificity. There are at least three distinct p110 catalytic subunits of Class I phosphoinositide 3-kinases: p110α, p110β, and p110δ. These proteins mediate binding to the regulatory subunit, which could serve as a mechanism for distinct location and/or activation of different pools of Akt to mediate differential signaling of nuclear transcription factors, allowing insulin to appropriately regulate some genes (GLUT2 and glucokinase), but not others (PEPCK and G6Pase). Decreased IRS-2 is an important component underlying increased PEPCK expression in hepatic insulin resistance, but does not affect the action of insulin to regulate other signaling pathways important for the transcriptional and acute post-transcriptional effects of insulin to suppress HGP. This model also suggests that hepatic insulin resistance involves modification of phosphorylation of certain transcription factors required for insulin-mediated gene expression. PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent kinase-1; GSK3, glycogen synthase kinase-3; PFK-1, phosphofructokinase-1; PEP, phosphoenolpyruvate; PK, protein kinase; OAA, oxaloacetate.

In summary, these findings provide strong evidence that a relatively small increase in PEPCK gene expression can trigger increased HGP and impaired glucose tolerance. These findings also suggest that the acute effects of insulin on suppression of HGP in the liver may occur through a mechanism independent of IRS-2, requiring post-transcriptional regulation of pathways other than gluconeogenic gene expression. Thus, increased
HGP and insulin resistance are likely due to selective effects of insulin on the liver to directly suppress hepatic gene expression as well as defects in other signaling pathways that mediate the metabolic effects of insulin. Although there is no evidence that regulation of PEPCk is the cause of hepatic insulin resistance in type II diabetes mellitus, these results clearly demonstrate that a defect in insulin regulation of hepatic gene expression is sufficient to cause a chronic increase in basal HGP and selective hepatic insulin resistance, independent of changes in peripheral insulin resistance or plasma FFAs.

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