Overexpression of Glutamine:Fructose-6-Phosphate Amidotransferase in the Liver of Transgenic Mice Results in Enhanced Glycogen Storage, Hyperlipidemia, Obesity, and Impaired Glucose Tolerance

Geddati Veerababu, Jiping Tang, Rosemary T. Hoffman, Marc C. Daniels, Leon F. Hebert Jr., Errol D. Crook, Robert C. Cooksey, and Donald A. McClain

To examine the effect of increased hexosamine flux in liver, the rate-limiting enzyme in hexosamine biosynthesis (glutamine:fructose-6-phosphate amidotransferase [GFA]) was overexpressed in transgenic mice using the PEPCK promoter. Liver from random-fed transgenic mice had 1.6-fold higher GFA activity compared with nontransgenic control littermates (276 ± 24 pmol · mg^{-1} · min^{-1} in transgenic mice vs. 176 ± 18 pmol · mg^{-1} · min^{-1} in controls, $P < 0.05$) and higher levels of the hexosamine end product UDP-*N*-acetyl glucosamine (288 ± 11 pmol/g in transgenic mice vs. 233 ± 10 pmol/g in controls, $P < 0.001$). Younger transgenic mice compared with control mice had lower fasting serum glucose (4.8 ± 0.5 mmol/l in transgenic mice vs. 6.5 ± 0.8 mmol/l in controls, $P < 0.05$) without higher insulin levels (48.0 ± 7.8 pmol/l in transgenic mice vs. 56.4 ± 5.4 pmol/l in controls, P = NS); insulin levels were significantly lower in transgenic males ($P < 0.05$). At 6 months of age, transgenic animals had normal insulin sensitivity by the hyperinsulinemic clamp technique. Hepatic glycogen content was higher in the transgenic mice (108.6 ± 5.2 µmol/g in transgenic mice vs. 32.8 ± 1.3 µmol/g in controls, $P < 0.01$), associated with an inappropriate activation of glycogen synthase. Serum levels of free fatty acids (FFAs) and triglycerides were also elevated (FFAs, 0.67 ± 0.03 mmol/l in transgenic mice vs. 0.14 ± 0.01 in controls; triglycerides, 1.34 ± 0.15 mmol/l in transgenic mice vs. 0.38 ± 0.01 in controls, $P < 0.01$). Older transgenic mice became heavier than control mice and exhibited relative glucose intolerance and insulin resistance. The glucose disposal rate at 8 months of age was 154 ± 5 mg · kg^{-1} · min^{-1} in transgenic mice vs. 191 ± 6 mg · kg^{-1} · min^{-1} in controls ($P < 0.05$). We conclude that hexosamines are mediators of glucose sensing for the regulation of hepatic glycogen and lipid metabolism. Increased hexosamine flux in the liver signals a shift toward fuel storage, resulting ultimately in obesity and insulin resistance. *Diabetes* 49:2070–2078, 2000

From the Division of Endocrinology, Department of Medicine, University of Utah School of Medicine, and the Veterans Administration Medical Center, Salt Lake City, Utah.

Address correspondence and reprint requests to Donald A. McClain MD, PhD, Department of Medicine, University of Utah, 50 N. Medical Dr., 4C116, Salt Lake City, UT 84132. E-mail: donald.mcclain@hsc.utah.edu.

Received for publication 10 November 1999 and accepted in revised form 22 August 2000.

FFA, free fatty acid; G6P, glucose-6-phosphate; GFA, glutamine:fructose-6-phosphate amidotransferase; IPGTT, intraperitoneal glucose tolerance test; PCR, polymerase chain reaction; RT, reverse transcriptase, UDP-Glc-NAc, UDP-*N*-acetyl glucosamine.

Glucose metabolism through the hexosamine biosynthesis pathway has been hypothesized to mediate many of the adverse effects of hyperglycemia and to be involved in the pathogenesis of type 2 diabetes and glucose toxicity (1–9). Adipocytes (2), fibroblasts (4,8), and intact rodents (5,6,9) exposed to glucosamine develop insulin resistance. The rate-limiting enzyme in the hexosamine biosynthesis pathway is glutamine:fructose-6-phosphate amidotransferase (GFA), which catalyzes the formation of glucosamine-6-phosphate from fructose-6-phosphate and glutamine. Like animals exposed to glucosamine, transgenic animals overexpressing GFA in skeletal muscle and fat also develop insulin resistance (7). This peripheral insulin resistance is very similar to that observed in type 2 diabetes: The transgenic animals exhibit decreased translocation of the GLUT4 glucose transporter, and the insulin resistance is reversed with the antidiabetic drug troglitazone (10). Less is known about the effects of hexosamines in humans, although GFA levels do correlate inversely with glucose disposal rate in humans (11) as they do in mice (7). GFA levels are also elevated in muscle from subjects with type 2 diabetes (12), although these changes may be secondary to regulation of GFA by glucose and insulin (11).

In addition to insulin resistance, defects in hepatic glucose regulation also play a key role in the pathogenesis of type 2 diabetes (13,14). If alterations in hexosamine synthesis and its downstream signaling pathways play a role in diabetes, it would be expected that overexpression of GFA in liver would lead to changes in fuel homeostasis reminiscent of type 2 diabetes, as was the case for overexpression of GFA in muscle and fat. We have therefore engineered and characterized transgenic mice that overexpress GFA in their livers. Their phenotype of obesity, glycogen accumulation, hyperlipidemia, and decreased glucose tolerance with aging is consistent with a role for hexosamines in glucose sensing and metabolic regulation. Chronic overactivity of this pathway results in many features of the insulin resistance syndrome (15).

RESEARCH DESIGN AND METHODS

Materials. Routine reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Transgenic animals. Transgene expression was targeted to liver tissue using the PEPCK promoter provided by Dr. Richard Hanson. The transgene consisted of 2,070 bp of the PEPCK promoter (–2,000 to +70) (16) linked to the human GFA cDNA (17,18) and the SV40 polyadenylation sequence. The construct was purified and microinjected into one-cell mouse embryos that were then surgically reimplanted into pseudopregnant female mice at the University of Alabama Transgenic Facility in Birmingham, Alabama. Heterozygous transgenic mice and control nontransgenic animals from the same litters were used in experiments that were approved by the laboratory animal use committees at the University of Utah Medical Center and the Veterans Affairs Medical Center in Jackson, Mississippi. Data from both male and female animals were pooled for analysis. Although several founders exhibited the obese phenotype, for consistency all of the reported experiments were performed on the offspring of a single founder male, that strain being designated PEPCK-GFA-1.

DNA and RNA analysis. Polymerase chain reaction (PCR) amplification of mouse tail chromosomal DNA was performed to verify the presence of the transgene in offspring of transgenic animals. Mouse tail DNA was extracted and amplified using sense and antisense primers that anneal to nucleotides 376–392 and 1025–1044 of the human GFA coding region, respectively, to yield a 669-bp product (17). These primers span six intron-exon boundaries in the endogenous GFA gene, thus ensuring specificity of the PCR products for the intronless transgene. PCR proceeded for 30 cycles of 94°C for 1 min, 42°C for 1 min, and 72°C for 2 min.

The presence of transgene-encoded mRNA was confirmed by reverse transcriptase (RT)-PCR followed by restriction digestion at a site unique to the human (transgenic) product. Animals were killed after an overnight fast, and liver and muscle were dissected and frozen in liquid nitrogen. Frozen tissues were pulverized under liquid nitrogen, the powder homogenized in Trizol reagent, and RNA isolated according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Some 5 µg of total RNA were used for first-strand synthesis using Ready-to-Go U-Prime Beads (Pharmacia, Piscataway, NJ) and an oligo dT primer. A specific GFA fragment was then amplified using primers hybridizing to nucleotides 89–115 (sense) and 1989–2016 (antisense) of the GFA cDNA. The 1.9-kb product was reamplified using nested primers (sense, 292–320 bp and antisense, 1,803–1,828 bp) at a 55°C annealing temperature to produce a 1.5-kb fragment. The human sequence but not the mouse sequence contains a unique *Hin*cII site at 1,032 bp, with cleavage yielding 739- and 796-bp fragments; any RT-PCR product derived from endogenous mouse GFA mRNA would not be digested by *Hin*cII. Products were analyzed on 1% agarose gels.

Assay of GFA activity. Both random-fed control and transgenic mice were killed and the livers were excised. A piece of liver (~0.1 mg) was immediately placed in 1.0 ml of extraction buffer (25 mmol/l Tris pH 7.5, 1.0 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l dithiothreitol, 200 µmol/l phenylmethylsulfonyl fluoride [PMSF], 2 µg/µl leupeptin, 1.0 µg/µl pepstatin, 1.0 µg/µl aprotinin, and 100 mmol/l NaF). The samples were then homogenized (VirTis homogenizer; VirTis, Gardiner, NY) for 10 s and sonicated (50 Sonic Dismembrator; Fisher Scientific, Tustin, CA) at setting 7 for 10 s at 4°C The samples were centrifuged at 60,000*g* for 15 min at 4°C, and the supernatant was assayed for GFA activity. GFA activity in ~10 µg of protein was assayed using a previously published procedure (7).

Glucose, insulin, free fatty acid, and triglyceride levels in serum. The following diagnostic kits were used: glucose (glucose oxidase method, Sigma), free fatty acids (FFAs) (acyl-CoA synthetase/acyl CoA oxidase/peroxidase method, Roche Diagnostics, Mannheim, Germany), and triglycerides (lipoprotein lipase/glycerol kinase/glycerol phosphate oxidase/peroxidase method, Sigma). Insulin concentrations were measured by using the Linco sensitive rat insulin radioimmunoassay kit (Linco Research, St. Louis, MO).

Glycogen content. The glycogen content in the liver extracts of control and transgenic mice was determined with amyloglucosidase according to the method of Keppler and Decker (21). Liver tissue previously harvested, frozen in liquid nitrogen, and stored at –80°C was weighed and homogenized as above with five parts by weight of ice-cold perchloric acid (0.6N) . After centrifugation for 15 min at 3,000*g*, 0.2 ml of the homogenate was incubated with 0.1 ml of 1 mol/l potassium hydrogen carbonate and 2.0 µl of 10 mg/ml amyloglucosidase with shaking at 40°C for 2 h. After adding 1.0 ml of 0.6N perchloric acid, the samples were centrifuged for 15 min and 100 µ1 acid supernatant was used for the determination of glucose in the presence of triethanolamine buffer (0.3 mol/l triethanolamine pH 7.5, 0.5 mmol/l $MgS0_4$, 1 mmol/l ATP, 0.9 mmol/l NADP, and 5 µg glucose-6-phosphate dehydrogenase) and 5 µl of 2 mg/ml hexokinase. The glucose liberated after hydrolysis of glycogen is proportional to the increase of NADPH measured by the extinction change at 340 nm. The glycogen content is expressed as micromoles per gram wet weight of tissue.

Glycogen synthase activity assay. The assay of glycogen synthase was a modification (3) of the procedure of Thomas et al. (22). Previously frozen liver tissue was homogenized in 20 volumes of assay buffer as described (3), and ~100–200 µg protein was assayed in the presence or absence of 4.7 mmol/l glucose-6-phosphate (G6P).

Phosphorylase activity. Assay of glycogen phosphorylase was modified from Gilboe et al. (23). The liver tissue was added to 100 volumes of ice-cold 50 mmol/l morpholineethanesulfonic acid (MES) buffer, pH 6.1, containing 50 mmol/l KF and 60 mmol/l 2-mercaptoethanol. The tissue was homogenized and centrifuged as for the glycogen synthase assay (3). Some 20 µl of the diluted homogenate was added to 40 µl of an assay mixture containing 200 mmol/l KF, 100 mmol/l [^{14}C]glucose-1-phosphate (~0.0025 µCi/µmol final specific activity), and 1% rabbit liver glycogen with (for total phosphorylase activity) or without (activated fraction) 3 mmol/l 5′-AMP. Incubation at 30°C was allowed to proceed for 30 min. At this time, a 50-µl aliquot was removed and spotted immediately on a 1-inch square of Whatman 3MM paper. The filter paper was held for 2 or 3 s after spotting to ensure complete absorption of the sample, then dropped into 66% ethanol and washed and counted as above.

G6P, UDP-glucose, and ATP. G6P was determined in the liver of control and transgenic mice by a modification of the method of Lang and Michal (24) using G6P dehydrogenase. The frozen liver samples (0.3 g) were homogenized in 2.5 ml of 6N perchloric acid and centrifuged at 3,000*g* for 10 min. The sediment was swirled in 1 ml perchloric acid and 1 ml distilled water and the contents were recentrifuged. The supernatants were combined and pH adjusted to pH 3.5 with potassium carbonate solution. The final volume of the sample was brought to 5 ml, allowed to stand in an ice bath for 15 min, and the supernatant was saved for assay. The G6P was assayed with 1 ml of the supernatant in the presence of 0.4 mol/l triethanolamine (pH 7.6), 20 mmol/l NADP, 0.5 mol/l $MgC1_2$, and 5 µg G6P dehydrogenase suspension. Absorbance was read at 340 nm in the spectrophotometer and the concentration of G6P determined by comparison to known standards.

UDP glucose was determined by the method of Keppler and Decker (21) using UDP glucose dehydrogenase. Frozen liver tissue (0.3 g) was homogenized in five parts by weight of ice-cold perchloric acid (0.6N). The homogenate was allowed to stand for 10–15 min at 0°C and then recentrifuged for 15 min at 10,000*g*. The supernatant (0.15 ml) was decanted and brought to pH 8.0 with potassium hydrogen carbonate. After it was allowed to stand for 15 min at 4°C, the sample was centrifuged and the supernatant assayed. The UDP glucose in 0.2 ml of the sample was assayed in the presence of 0.35 mol/l glycine, 2.1 mmol/l NAD, 5.6 mmol/l EDTA, and 0.02 ml of UDP glucose dehydrogenase suspension. Absorbance was read at 340 nm in the spectrophotometer and the concentration of UDP glucose determined by comparison to known standards.

ATP in tissue was measured using the phosphoglyceric phosphokinase-glyceraldehyde phosphate dehydrogenase method (Sigma).

UDP-*N*-acetyl-glucosamines. Levels of UDP-*N*-acetyl glucosamine (UDP-GlcNAc) were measured as described by Robinson et al. (25). Liver (~0.2 g) from random-fed control and transgenic mice was homogenized at 4°C in 4 volumes of perchloric acid (300 mmol/l) and centrifuged (10,000*g* for 15 min at 4°C). Acid was extracted from the supernatants with 2 volumes of tri-n-octylamine:1,1,2-trichlorofluoroethane (1:4). The aqueous phase was filtered (0.45 µm) and high-performance liquid chromatography was performed on a Spherisorb amino column (25 cm × 4.6 mm., Phase Separations) eluted with a concave gradient from 80 mmol/l potassium phosphate (pH 2.8), 35% acetonitrile, to 800 mmol/l potassium phosphate (pH 3.6) over 48 min at flow rate of 1 ml/min (26). UDP-GlcNAc levels were quantified by ultraviolet absorption at 254 nm, compared with external standards, and normalized to tissue weight.

Glucose tolerance test. After an 18-h fast, a glucose load of 1 mg/g body wt was administered intraperitoneally. Tail vein blood was sampled for blood glucose determination (Miles Elite glucometer) from nonsedated animals before and at 15, 30, 60, 90, and 120 min after glucose administration.

Determination of glucose disposal rates. All experiments were performed in weight-matched nonsedated transgenic and littermate control mice using the hyperinsulinemic-euglycemic clamp technique previously described (7). Catheters were implanted into the right internal jugular vein. The animals were allowed to recover from surgery for 3 days and were fasted overnight before the experiment. Animals were infused with recombinant human insulin (Humulin; Eli Lilly, Indianapolis, IN) at a rate of 20 $mU \cdot kg^{-1} \cdot min^{-1}$ while 50% dextrose was infused by a variable infusion pump (Harvard Apparatus, South Natick, MA). Whole-blood samples (3 ul) were collected every 5–10 min from tail bleeds and measured by glucometer.

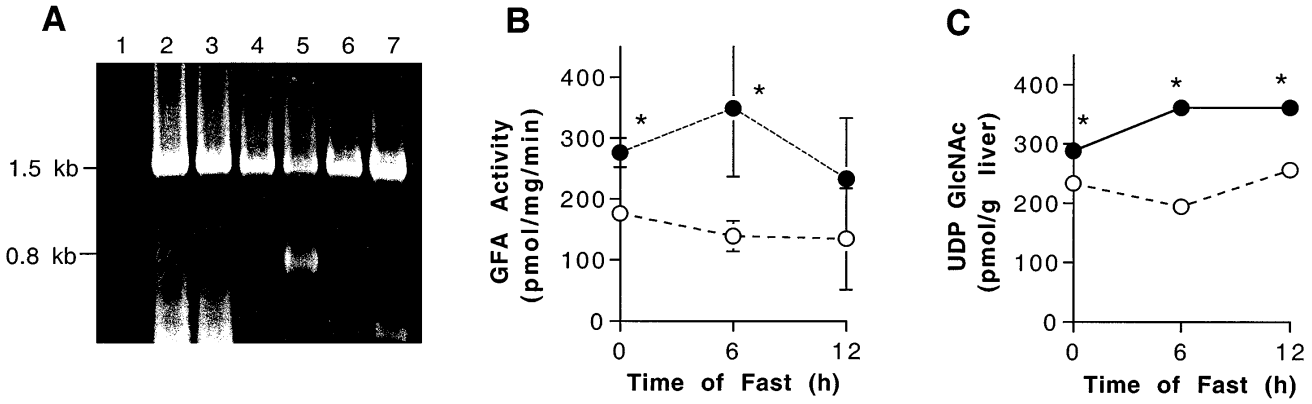

FIG. 1. Agarose gel electrophoresis of RT-PCR products with and without *Hin*cII digestion. *A*: *Lane 1*: No RT. Mouse liver RNA was PCR amplified but without the RT step. The entire reaction was precipitated, dissolved in 10 mmol/l Tris, 1 mmol/l EDTA, and one-quarter of the product was loaded on the gel. *Lanes 2–7*: 1 ng of each RT-PCR product was reamplified using nested primers. Aliquots of the product were incubated for 2 h at 37°C with buffer alone or with *Hin*cII. *Lanes 2 and 3*: control liver; *lanes 4 and 5*: PEPCK-GFA liver; *lanes 6 and 7*: PEPCK-GFA muscle. DNA in *lanes 2, 4*, and *6* incubated with buffer alone; DNA in *lanes 3, 5*, and *7* was digested with *Hin*cII. *B*: Liver GFA activity in control and PEPCK-GFA-1 mice. Results are from *n* = 12 each control and transgenic livers, with GFA activity assayed in vitro as described. *C*: Liver UDP-GlcNAc content in control and PEPCK-GFA-1 mice. Results are from *n* = 12 each control and transgenic livers, expressed as means ± SD.

RESULTS

Overexpression of GFA in livers of transgenic mice. We first verified that the animals overexpressed GFA in the liver. Founder transgenic animals were selected that had integrated the transgene into their genome as demonstrated by the presence of a transgene-specific PCR product after amplification of genomic DNA (not shown). In one founder, transcription of the transgene was verified by RT-PCR. Liver mRNA was reverse transcribed and amplified using oligonucleotide primers that would lead to the generation of a 1.5-kb fragment. Amplification of mRNA without the RT resulted in no product (Fig. 1*A*, *lane 1*), confirming that the products observed in *lanes 2–7* were not due to contaminant DNA in the RNA preparation. The DNA fragment resulting from the PCR was then digested with the restriction enzyme *Hin*cII. Digestion with *Hin*cII of a PCR fragment resulting from amplification of human GFA cDNA would be predicted to lead to fragments of DNA of sizes 739 and 796 bp; a PCR fragment resulting from the amplification of endogenous mouse GFA would not be digested by *Hin*cII. As shown in *lanes 2–5*, the RT-PCR fragment from transgenic mice but not from control mice was digested by *Hin*cII, confirming the presence of human (transgenic) mRNA for GFA in the liver of a mouse line designated as PEPCK-GFA-1. Undigested DNA was also present, due to the amplification of mouse GFA mRNA whose sequence shares high homology with that of humans (27). Skeletal muscle from line PEPCK-GFA-1 did not express transgenic GFA as shown in *lanes 6* and *7*. Although RT-PCR amplification of muscle mRNA did lead to generation of a product, this product is endogenous mouse GFA because it did not contain the *Hin*cII site (*lane 7*).

GFA activity. GFA enzymatic activity was elevated in the livers of the PEPCK-GFA transgenic mice. As shown in Fig. 1*B*, random-fed transgenic mice had 1.6 times the GFA activity in liver homogenates compared with nontransgenic littermates ($P < 0.05$, $n = 12$). Because the PEPCK promoter/enhancer driving GFA expression is regulated by fasting/feeding, we also investigated the effect of fasting on GFA activity. GFA activity in transgenic animals increases further after food withdrawal, resulting in a 2.5-fold increase over that of control animals after 6 h of fasting ($P < 0.01$, $n = 5$). After 12 h of fasting, GFA levels in transgenic mice were 1.7-fold higher, but this difference as measured in a small sample of animals did not reach statistical significance ($P = 0.06$, $n = 3$). Levels of GFA activity did not differ between control and transgenic animals in fat or muscle tissue (not shown).

UDP-GlcNAc. The principal end products of the hexosamine pathway are UDP-GlcNAc and other UDP-*N*-acetyl-hexosamines, and these compounds have been shown to reliably reflect hexosamine biosynthetic pathway activity and to correlate well with the biologic consequences of increased hexosamine flux (6,7,10). UDP-GlcNAc levels were significantly elevated 1.2-fold in the livers of random-fed transgenic animals (Fig. 1*C*, $P < 0.001$). Further increases of UDP-GlcNAc were seen in transgenic animals after fasting, 1.9-fold at 6 h ($P < 0.001$, $n = 13$) and 1.4-fold at 12 h ($P < 0.001$, $n = 5$). Glucosamine-6-phosphate levels were 1.5-fold higher in transgenic liver ($P < 0.05$), but the levels of several other metabolic intermediates (UDP glucose, G6P, and fructose-6-phosphate) were not significantly altered (Table 1).

General characteristics of PEPCK-GFA-1 mice. PEPCK-GFA-1 mice were originally developed using oocytes fertilized with sperm from F1 hybrid C57BL6/SJL mice. As shown in Fig. 2, the PEPCK-GFA-1 founder male was obese, weighing 52.8 g at the age of 14 months. In the first litter, the het-

TABLE 1
Levels of metabolic intermediates in livers of mice overexpressing GFA

Metabolic intermediate	Controls	Transgenic mice
Glucosamine-6-phosphate (nmol/l)	0.34 ± 0.01	0.51 ± 0.03*
UDP-glucose (mmol/l)	0.27 ± 0.01	0.33 ± 0.02
Fructose-6-phosphate (mmol/l)	1.19 ± 0.08	1.25 ± 0.07
G6P (mmol/l)	1.48 ± 0.09	1.75 ± 0.09

Data are means ± SE for nine determinations in each group. *$P < 0.05$ by Student's *t* test on animals paired by age and sex.

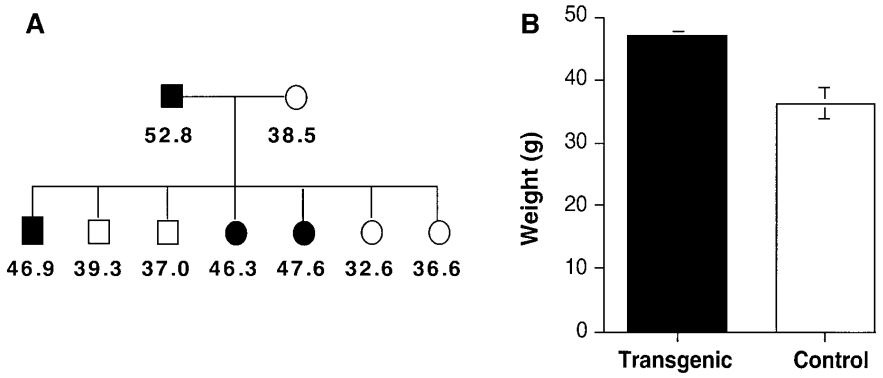

FIG. 2. Weights of PEPCK-GFA-1 mice. PEPCK-GFA-1 founder male was obese, weighing 52.8 g at the age of 14 months. *A*: Pedigree of the first generation is shown. Solid figures represent transgene-positive animals, with weights in grams at ages 12–13 months below each figure. *B*: The combined weights of transgenic offspring compared with their nontransgenic littermates ($P < 0.05$).

erozygous offspring carrying the transgene were 29% heavier than their nontransgenic littermates ($P < 0.05$). With subsequent breeding into the line SJL, however, the obesity phenotype became less distinct, so after more than five generations of breeding into SJL, younger transgenic mice (<12 months old) were no heavier than their nontransgenic littermates and in the group >12 months old were only 13% heavier than the controls, although the latter difference was significant when males and females were considered separately (Table 2, $P < 0.05$). More recent backbreeding into C57BL6 mice, a strain more susceptible to the expression of diabetic and obese traits (28), has resulted in the reemergence of more pronounced obesity as a phenotype of the transgenic mice (data not shown).

Blood chemistries and glucose tolerance of transgenic mice. Blood chemistries from transgenic mice and littermate controls are shown in Table 2. In transgenic mice <12 months old, the average fasting blood glucose was 26% lower ($P < 0.05$). Fasting insulin levels were 16% lower than in age-matched littermates; this difference was not significant for the mice considered in aggregate ($P = 0.07$, NS), although the transgenic males did have significantly lower insulin levels (44.5 ± 4.3 vs. 58.8 ± 2.9 pmol/l, $P < 0.05$). In a group of mice >12 months old, the fasting glucose and insulin levels were 6 and 20% higher, respectively, than those in control mice of the same age, but the latter difference was not statistically significant. Liver weights did not differ between control and transgenic mice.

To explore further the changes in glucose tolerance with aging, we performed intraperitoneal glucose tolerance tests (IPGTTs) on a cohort of littermates. As seen in Fig. 3*A*, there were changes in glucose tolerance with aging. Consistent with results in Table 2, the younger transgenic mice had lower glucose excursions with IPGTT than their control littermates. Once mice were 10 months old, however, this situation had reversed, and by 13–14 months of age, the transgenic mice had a 12.5% increase in the area under the curve with IPGTT compared with controls. The age-related changes in the areas under the curve are significant ($P < 0.001$ by analysis of variance). The weights of the mice used in this longitudinal study are shown in Fig. 3*B*. These weights are significantly correlated with the glucose area under the curve ($r^2 = 0.735$, $P < 0.01$) and that relationship holds and is independent of whether the mice carry the transgene or not.

Consistent with the glucose tolerance data, the transgenic animals did develop insulin resistance as they aged. As seen in Fig. 4, in 6-month-old mice the glucose disposal rates measured during a hyperinsulinemic clamp did not differ between control and transgenic mice. By 8 months, however, the transgenic animals were significantly insulin resistant, with a 20% decrease in glucose disposal rate ($P < 0.05$).

Hepatic glycogen content in PEPCK-GFA-1 mice. We and others have hypothesized that hexosamines may be used by tissues to sense net glucose flux, thus allowing appropriate responses by that tissue in the presence of changes in substrate availability (1,2,6). Because hepatic glycogen flux is tightly regulated by glucose, we sought to determine whether that metabolic pathway might be regulated through hexosamines. We first determined whether there were any changes in net glycogen accumulation in the transgenic animals. As shown in Fig. 5, glycogen content was 3.2-fold higher in random-fed PEPCK-GFA-1 animals aged 2–4 months compared with nontransgenic littermates ($P < 0.01$). Upon fasting, glycogen in both control and transgenic animals was nearly completely mobilized.

Glycogen synthase activity in PEPCK-GFA-1 mice. Net glycogen accumulation is a function of glycogen synthesis and glycogenolysis, these reactions being regulated chiefly by the activation states of the enzymes glycogen synthase and phosphorylase. We therefore determined the activities of these enzymes as a function of time of fasting in extracts of the same livers used above to determine glycogen content. The activity of glycogen synthase is shown in Fig. 6. Total glycogen synthase activity (Fig. 6*A*) measured in the presence of a maximally activating concentration of G6P did not differ between the control and transgenic animals. Although the total activity

TABLE 2
Characteristics of mice overexpressing GFA in liver

	Controls	Transgenic mice
Mice <12 months old		
Weight (g)	23.4 ± 0.7	23.1 ± 1.3
Fasting glucose (mmol/l)	6.5 ± 0.8	4.8 ± 0.5*
Fasting insulin (pmol/l)	56.4 ± 5.4	48.0 ± 7.8
Liver weight (g)	1.0 ± 0.2	1.1 ± 0.1
Mice >12 months old:		
Weight (g)	26.6 ± 2.3	30.1 ± 0.8*†
Fasting glucose (mmol/l)	7.9 ± 1.1	8.4 ± 0.8†
Fasting insulin (pmol/l)	51.0 ± 7.2	61.2 ± 5.4

Data are means ± SE for nine determinations in each group. Plasma glucose and insulin levels were measured as described after an overnight fast. *$P < 0.05$ by Student's *t* test on control vs. transgenic mice. †$P < 0.05$, older vs. younger mice.

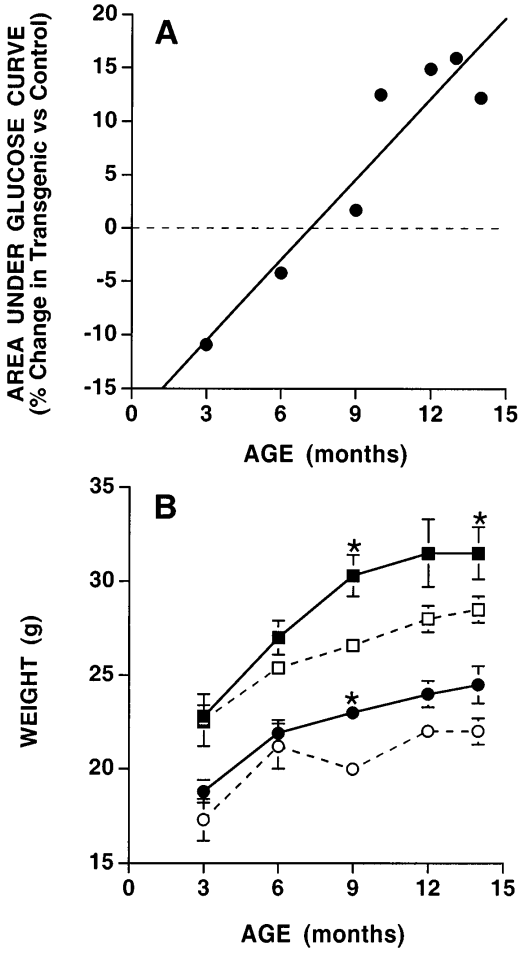

FIG. 3. Changes in glucose tolerance with aging using IPGTTs. *A*: Changes in glucose tolerance with aging. The younger transgenic mice had lower glucose excursions with IPGTT than their control littermates. However, after 9 months, the transgenic mice had a 12–16% increase in the area under the curve with IPGTT. *B*: Weights of the mice used in this longitudinal study (■, transgenic male; ●, transgenic female; □, control male; ○, control female. n = 4–5 per group, * P < 0.05 for control vs. transgenic within sex).

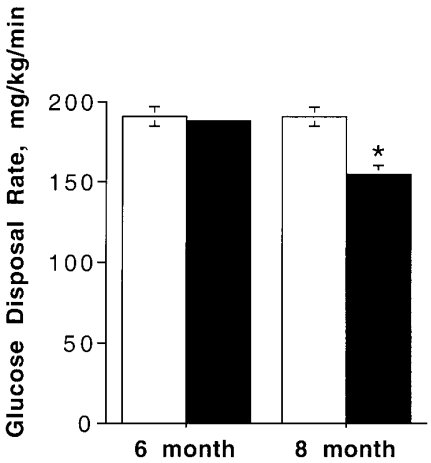

FIG. 4. Glucose disposal rates in control (□) and PEPCK-GFA-1 transgenic (■) mice. Glucose disposal was measured by the hyperinsulinemic clamp technique in mice of the indicated ages (*P < 0.05 compared with controls of the same age, n = 3 control and 4 transgenic animals).

varied over the course of the 12-h fast, the changes over time were not significantly different from baseline.

The fractional activation of glycogen synthase (velocity of synthase measured in the absence of G6P and expressed as the percent of total activity) did differ in the transgenic animals, however (Fig. 6*B*). In the fed animals, activated synthase was significantly lower in the transgenic animals (P < 0.01). With feeding, the fractional activation of synthase declined slowly in the control animals as their liver glycogen was depleted. In contrast, the fractional activation in transgenic animals increased and was nearly threefold higher than that in controls by the end of the 24-h fast (17.6 ± 3.9 vs. 6.4 ± 1.0 in transgenic versus control mice, P < 0.05).

One possible explanation for the variation in glycogen synthase activity is the presence of allosteric activators in the reaction mixture. This was not the case, because total and fractional synthase activities were found to be linear and directly proportional to protein concentration over a 100-fold range of dilution of the hepatic extracts (data not shown). If the differences between control and transgenic enzyme activities were due to small molecule activators in the extract, the fractional activity of synthase and the relative differences between control and transgenic activities would decrease with dilution as the concentration of the putative activator decreased. Furthermore, levels of the allosteric activators G6P and glucosamine-6-phosphate do not account for the observed increase in activity (Table 1).

Glycogen phosphorylase activity in PEPCK-GFA-1 mice. Figure 6 also shows the activity of glycogen phosphorylase as a function of time of fasting in the control and transgenic mice. In Fig. 6*C*, one can see that the level of total phosphorylase was higher in the transgenic animals (P < 0.01 over all times) and that the levels did not vary significantly over the fasting period. The fractional activation of phosphorylase did not differ between control and transgenic animals and did not vary during the fast (Fig. 6*D*).

Serum free fatty acid and triglyceride levels in PEPCK-GFA-1 mice. Figure 7 shows that serum free fatty acid (FFA) and triglyceride levels were 4.9-fold and 3.5-fold elevated in random-fed transgenic mice (FFA, 0.67 ± 0.03 vs. 0.14 ± 0.01 mmol/l in controls, P < 0.01; triglyceride, 1.34 ± 0.15 vs. 0.38 ± 0.01 mmol/l in controls, P < 0.01). These measurements, like those of glycogen content, were made in 2- to 4-month-old animals, before insulin resistance or glucose intolerance had developed.

ATP levels in liver of control and transgenic mice. Because of the concern that in vitro treatments with high concentrations of glucosamine may lead to ATP depletion, we measured ATP levels in the liver of control and transgenic mice. However, there was no evidence of ATP depletion in the transgenic animals with controls containing 14.3 ± 0.6 and transgenic mice 16.6 ± 1.2 µmol/mg tissue (mean ± SE for six determinations from six different livers in each group).

DISCUSSION

In summary, mice overexpressing GFA in liver have increased hepatic glycogen content and FFA and triglyceride levels, and as they age, they become overweight and exhibit decreased glucose tolerance. The changes in glycogen and lipids are consistent with a role of hexosamines in glucose sensing in the liver. In younger transgenic animals

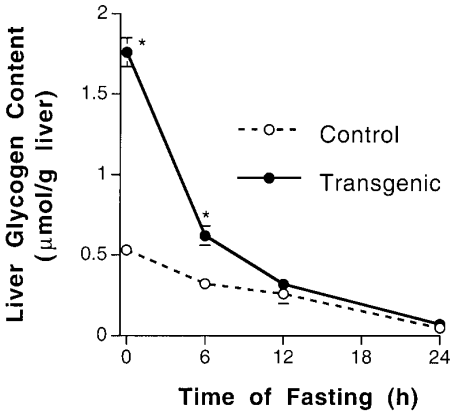

FIG. 5. Hepatic glycogen content in control and PEPCK-GFA-1 mice. Glycogen content was measured in random-fed animals and after the indicated times of fasting in random-fed PEPCK-GFA-1 animals (●) compared with nontransgenic littermates (○). Glycogen content was 3.2-fold elevated in random-fed PEPCK-GFA-1 animals (n = 9 animals per data point, *P < 0.01 compared with controls).

overexpressing GFA, there is significantly greater hepatic glycogen accumulation despite decreased blood glucose levels and concomitant decreases in insulin levels. Thus, the hepatic response to increased hexosamine flux in these animals is the same as if there were high glucose flux, with the animals shifting inappropriately toward fuel storage even in the presence of relative hypoglycemia.

The glycogen accumulation is not pathologic: The degree of hepatic glycogen accumulation is in the normal physiological range (29), and the glycogen is mobilized normally during a fast. The molecular mechanisms underlying these changes include an increase in the fractional activation state of glycogen synthase. The change in synthase activity probably involves posttranslational regulation, because we did not see any change in the total amount of glycogen synthase activity measured in the presence of maximally activating concentrations of G6P. The activation of synthase may be the chief explanation for excess glycogen accumulation in the liver, although total glycogen accumulation is known not to be a simple function of the activities of glycogen synthase and phosphorylase as measured in vitro (30). Other factors such as the nature and size of the glycogen particles (31), the state and amount of glycogenin (32), the subcellular partitioning of synthase (33), and the partitioning and cycling of its substrates (34) have also been implicated in the determination of net glycogen storage. Decreased synthase activity in the fed state may be due to excess glycogen accumulation (35,36) and thus be only indirectly related to the expression of the trans-

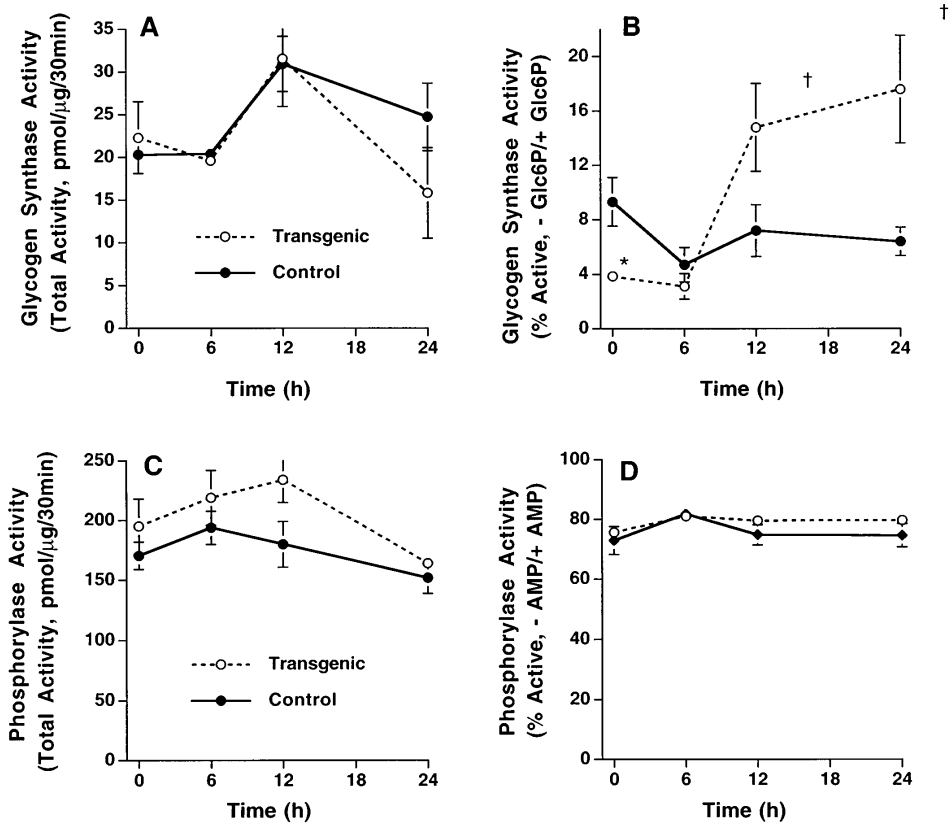

FIG. 6. Liver glycogen synthase and phosphorylase activities in PEPCK-GFA-1 mice as a function of time of fasting in the control (●) and transgenic (○) mice. *A*: Total glycogen synthase activity measured in the presence of G6P. At the indicated times, the mice were killed and glycogen synthase activity was measured in the liver of control and transgenic mice. The total activity varied over the course of the 12-h fast, but the changes were not significantly different from baseline. *B*: The fractional velocity of glycogen synthase measured in the absence of G6P. In the random-fed animals (time = 0), activated synthase was significantly lower in the transgenic mice (P < 0.01). The fractional activation increased and was nearly threefold higher in the transgenic animals by the end of the 24-h fast. *C*: Total glycogen phosphorylase activity measured in the presence of AMP was higher in the transgenic mice (P < 0.01); the levels did not vary significantly over the fasting period. *D*: The fractional activation of phosphorylase measured in the absence of AMP did not differ between control and transgenic animals. (Shown are means ± SE, n = 5 animals for each data point, *P < 0.01, †P < 0.05, transgenic mice compared with controls.)

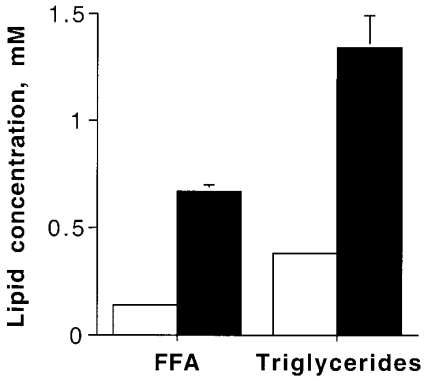

FIG. 7. FFA and triglyceride levels in blood samples of random-fed control (□) and transgenic (■) mice. FFA and triglyceride levels were 4.9-fold and 3.5-fold elevated in transgenic compared with control mice, respectively. Mice were aged 2–4 months. Results are the means ± SE of nine determinations, each from a separate animal. *P* < 0.01 for differences between control and transgenic animals for each lipid.

gene. Studies using hepatocytes cultured under controlled conditions are under way to further explore these questions.

Phosphorylase activities were modestly elevated in the transgenic mice, possibly accounting for the more rapid decline of glycogen with fasting. Unlike the case for glycogen synthase, the increase in phosphorylase was of total activity rather than in the activation state of the enzyme. Whether this is a direct effect of hexosamine flux or is secondary to the other metabolic changes seen in the transgenic animals remains to be determined. The more rapid glycogenolysis observed in these animals is similar to that seen in diabetic rats, where glycogenolysis is inappropriately maintained despite higher glucose fluxes (37). Overall hepatic glycogen metabolism in the PEPCK-GFA model may be similar to that seen in type 2 diabetes, an important pathophysiological component of which is altered glucose autoregulation (14). Although most studies of glycogen synthesis in type 2 diabetic tissues have examined skeletal muscle, it has been noted that liver glycogen stores are preserved in type 2 diabetes despite increased hepatic glucose output (38,39). Furthermore, increased hepatic glycogen stores may contribute directly to impaired hepatic autoregulation and increased hepatic glucose output (40).

The transgenic animals also develop hypertriglyceridemia, presumably as the glycogen-replete liver increases fatty acid synthesis and triglyceride export to allow long-term storage of the perceived excess fuel. The mechanisms for the increased FFA and triglyceride levels are unknown, but include at least in part upregulation of fatty acid synthase by hexosamines (S. Marshall, personal communication). These changes in glycogen and lipids precede the development of obesity and insulin resistance, suggesting that obesity and insulin resistance result from changes in patterns of fuel storage rather than vice versa.

A long-term consequence of the hexosamine-induced shift toward fuel storage is obesity in the transgenic animals. This phenotype was seen in three of the original founder mice generated from independent injections of the transgene into oocytes (not shown). This finding makes it extremely unlikely that the observed phenotype is a consequence of a local gene insertion effect as opposed to overexpression of GFA. The obesity is somewhat strain dependent, being less evident in SJL and more evident in the C57BL6 strain, known to be more susceptible to the expression of diabetic and obese phenotypes (28).

The younger transgenic animals (<12 months of age) had lower blood glucose concentrations and appropriately lower insulin levels. These insulin and glucose concentrations were in the physiological range, and insulin-to-glucose ratios were unchanged in the transgenic animals, arguing against the presence of a major defect in insulin action. Consistent with this finding, glucose clamp studies in the younger animals (Fig. 4) do not reveal changes in glucose disposal rates or hepatic glucose output rates. Thus, the young mice overexpressing GFA in liver are not significantly insulin resistant. Although the major phenotype observed in mice overexpressing GFA in skeletal muscle was insulin resistance (7), we do not view these data as contradictory. First, as the PEPCK-GFA animals age, their phenotype changes and they become insulin resistant, although it is likely that this is a secondary consequence of the effects of obesity and hyperlipidemia on muscle and/or the β-cell. More importantly, however, we believe that both model systems confirm the significance of hexosamines as physiological glucose sensors and not necessarily as mediators of pathology. For example, we have argued that the downregulation of insulin-stimulated glucose transport in muscle tissue induced by hyperglycemia or excess hexosamine flux is, in fact, autoregulatory and serves a protective function for muscle, normalizing net glucose flux into that tissue (1). Muscle tissue, by virtue of its ability to regulate glucose uptake, can therefore protect itself from excessive glucose flux, although the downstream consequences for the entire organism may eventually be detrimental.

As the PEPCK-GFA animals age, they become insulin resistant and glucose intolerant. This acquired defect in glucose disposal may be secondary to their excessive weight gain engendered by the shift toward energy storage signaled by the transgene. In fact, the area under the glucose curve is well correlated with the weight of the mice independent of sex and whether the mice carry the transgene. Increased levels of FFAs may also play a role, although these are seen in mice as young as 2 months old, long before there is any evidence of insulin resistance.

The increased FFA and triglyceride levels in the transgenic mice demonstrate that the hexosamine pathway not only regulates carbohydrate metabolism but fat metabolism as well. Conversely, it has also been demonstrated that increased fatty acid metabolism results in increased UDP-GlcNAc levels (41). The data are consistent with a role for the hexosamine pathway as a general "satiety sensor" for lipid as well as carbohydrate metabolism, signaling a shift in fuel utilization toward storage when hexosamine flux is increased.

This model of increased hexosamine flux is an inducible one. The PEPCK promoter was originally chosen for two reasons. First, we sought physiologic-range changes in hexosamine fluxes, and it was hoped that the promoter would lead to relatively modest levels of GFA overexpression and limited expression during embryogenesis. The variable levels of GFA are not crucial to the interpretation of the current study. Most important, the increased GFA and hexosamine levels are manifest even in the fed state when PEPCK expression is at its lowest, so the increased hexosamine flux is chronic. In previous studies, the changes in glycogen synthesis

in response to overexpression of GFA in fibroblasts occur in the time frame of 6–12 h, indicating that hour-to-hour fluctuations in GFA may not be as important as longer-term integrated hexosamine flux (3,4,8). Thus, the current data are consistent with our hypothesis that the hexosamine pathway serves as a sensor of chronic rather than acute satiety.

Chronic transgenic models of GFA overexpression are potentially less prone to artifact than studies using acute nonphysiological infusions of glucosamine. That is, with the relatively small increases in hexosamine levels that are in the range of physiological variation, changes in metabolic intermediates such as G6P, fructose-6-phosphate, and UDP glucose are not observed (Table 1). In contrast, treatment with glucosamine can exert effects on UDP glucose levels in muscle (42) or G6P levels in liver (43). Of particular concern is the potentially confounding effect of glucosamine treatment on cellular ATP levels (44). In the current study, hepatic levels of ATP were not altered in the transgenic animals, and the same has been shown to be true in transgenic animals overexpressing GFA in muscle and fat (10). ATP depletion is also not observed in animals infused with glucosamine (45,46). We feel that studies using GFA overexpression and, in most cases, acute glucosamine treatment do mirror the normal pathophysiology of hyperglycemia. This conclusion is reinforced by the observation that GFA activity is correlated with insulin resistance in non–experimentally manipulated humans (11,12).

The parallels between type 2 diabetes and the models of GFA overexpression in liver or in muscle plus fat (7,10) are striking, with both being characterized by insulin resistance, age-related obesity, and hypertriglyceridemia. We have also recently demonstrated that animals overexpressing GFA in the β-cell exhibit hyperinsulinemia and insulin resistance (unpublished observations). The parallels are noteworthy with the "thrifty phenotype" that was originally postulated to explain the high prevalence of type 2 diabetes, especially in populations that have historically faced periodic famine (47). The characteristics of the mice overexpressing GFA are consistent with this phenotype: In young animals, energy is stored at the expense of immediate access to that energy, i.e., lower blood sugar levels. Although this may be adaptive in situations of periodic calorie deprivation, with ad libitum feeding the consequences are obesity and diabetes. Further studies of these animals may aid in clarifying the relationships among the characteristics of the insulin resistance syndrome and type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (DK-43526), the Research Service of the Department of Veterans Affairs, and the Margolis Foundaton.

We thank Dr. Richard Hanson for his gift of the PEPCK promoter.

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