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Perturbation of Fuel Homeostasis Caused by Overexpression of the Glucose-6-phosphatase Catalytic Subunit in Liver of Normal Rats*

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The terminal step in hepatic gluconeogenesis is catalyzed by glucose-6-phosphatase, an enzyme activity residing in the endoplasmic reticulum and consisting of a catalytic subunit (glucose-6-phosphatase (G6Pase)) and putative accessory transport proteins. We show that Zucker diabetic fatty rats (fa/fa), which are known to exhibit impaired suppression of hepatic glucose output, have 2.4-fold more glucose-6-phosphatase activity in liver than lean controls. To define the potential contribution of increased hepatic G6Pase to development of diabetes, we infused recombinant adenoviruses containing the G6Pase cDNA (AdCMV-G6Pase) or the β-galactosidase gene into normal rats. Animals were studied by one of three protocols as follows: protocol 1, fed *ad libitum* for 7 days; protocol 2, fed *ad libitum* for 5 days, fasted overnight, and subjected to an oral glucose tolerance test; protocol 3, fed *ad libitum* for 4 days, fasted for 48 h, subjected to oral glucose tolerance test, and then allowed to refeed overnight. Hepatic glucose-6-phosphatase enzymatic activity was increased by 1.6–3-fold in microsomes isolated from AdCMV-G6Pase-treated animals in all three protocols, and the resultant metabolic profile was similar in each case. AdCMV-G6Pase-treated animals exhibited several of the abnormalities associated with early stage non-insulin-dependent diabetes mellitus, including glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content, and increased peripheral (muscle) triglyceride stores. These animals also exhibited significant decreases in circulating free fatty acids and triglycerides, changes not normally associated with the disease. Our studies show that overexpression of G6Pase in liver is sufficient to perturb whole animal glucose and lipid homeostasis, possibly contributing to the development of metabolic abnormalities associated with diabetes.

The liver has a large capacity for glucose production from gluconeogenesis and glycogenolysis, thereby providing protection against hypoglycemia. Production of free glucose via these pathways requires hydrolysis of glucose 6-phosphate, a step catalyzed by the glucose-6-phosphatase enzyme complex. Genetic and biochemical evidence suggests that the complex consists of the glucose-6-phosphatase catalytic subunit (G6Pase)[1] that resides in the lumen of the endoplasmic reticulum and putative endoplasmic reticulum membrane-localized glucose 6-phosphate, glucose, and inorganic phosphate transporter activities (1–6). Of these, only the G6Pase catalytic subunit and a potential glucose-6-phosphate translocase have been cloned (7–10).

Hepatic expression of G6Pase is increased, whereas levels of the glucose phosphorylating enzyme glucokinase are decreased, when diabetes is induced by partial pancreatectomy or streptozotocin administration (12–16). It has also been shown that metabolic abnormalities associated with NIDDM such as hyperglycemia and hyperlipidemia enhance hepatic expression of G6Pase (14, 16–18). Based on these findings, it has been suggested that perturbation of the balance between glucose phosphorylation and glucose 6-phosphate hydrolysis might contribute to the abnormal regulation of hepatic glucose production that is characteristic of non-insulin-dependent diabetes mellitus (NIDDM).

Although the foregoing observations are important, they do not provide direct evidence for a role of altered hepatic G6Pase expression in regulation of fuel homeostasis, for two main reasons. First, it cannot be assumed that altered expression of the G6Pase catalytic subunit alone will be sufficient to increase flux throughout the entire G6Pase complex. To partially test this point, we have recently used recombinant adenovirus technology to overexpress the G6Pase catalytic subunit in INS-1 insulinoma cells or primary rat hepatocytes, resulting in increased glucose 6-phosphate hydrolysis in both cell preparations (19, 20). In hepatocytes, overexpression of G6Pase resulted in marked lowering of glucose 6-phosphate levels and attendant decreases in glycogen synthesis and glycolytic flux (20). Whereas these data make it clear that flux through the glucose-6-phosphatase enzyme complex can be altered by manipulation of the expression level of the G6Pase catalytic subunit *in vitro*, it remains to be determined whether the results achieved in cultured cells can be replicated *in vivo*. The second major concern is that maneuvers that have been used to modulate G6Pase expression *in vivo* such as partial pancreatectomy, glucose infusion, or intralipid infusion can be expected to influence expression of a wide spectrum of genes encoding metabolic enzymes or other proteins (21), making it difficult to isolate the specific impact of G6Pase overexpression.

The purpose of this study was to address these issues and to

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[1] The abbreviations used are: G6Pase, glucose-6-phosphatase; AdCMV-G6Pase, recombinant adenovirus containing the cDNA encoding the catalytic subunit of glucose-6-phosphatase; ZDF, Zucker diabetic fatty; NIDDM, non-insulin-dependent diabetes mellitus; β-Gal, β-galactosidase; OGTT, oral glucose tolerance test; FFA, free fatty acids; TG, triglycerides.

evaluate the potential role of hepatic overexpression of the G6Pase catalytic subunit in the development of diabetes. In an initial set of experiments, we provide the first demonstration that hepatic G6Pase enzymatic activity is significantly elevated in a genetic model of obesity and diabetes, the Zucker diabetic fatty (ZDF) rat (fa/fa), relative to lean controls. To assess the potential contribution of up-regulated G6Pase expression to development of diabetes and obesity, we have used a recombinant adenovirus containing the G6Pase cDNA to overexpress the enzyme in liver of normal rats. Metabolic analyses performed on these animals under a variety of conditions demonstrate that they develop glucose intolerance and hyperinsulinemia, a marked decrease in hepatic glycogen levels and circulating hypolipidemia associated with tissue fat overstorage. These results demonstrate that up-regulated expression of the G6Pase catalytic subunit in liver alters a wide spectrum of metabolic parameters.

MATERIALS AND METHODS

Preparation of Recombinant Adenoviruses—A recombinant adenovirus containing the cDNA encoding the catalytic subunit of glucose-6-phosphatase (AdCMV-G6Pase) was prepared as described previously (19). A virus containing the *Escherichia coli* β-galactosidase gene (AdCMV-β-Gal; see Ref. 22) was used for control experiments.

Animal Experiments—Male Wistar rats (Charles River), Zucker diabetic fatty (ZDF) rats (fa/fa), and ZDF lean controls were used in these studies. ZDF obese and lean animals were studied at 9–10 weeks of age. Animals were housed individually and given free access to laboratory chow and water, except where indicated below, and food intake and body weight were recorded on a daily basis. In an initial set of experiments, liver samples were collected from all three rat strains for measurement of hepatic levels of G6Pase protein and enzymatic activity, using methods described below. In a subsequent series of experiments, AdCMV-G6Pase or AdCMV-β-Gal viruses were infused into normal Wistar rats, weighing 250–300 g. To reduce immunologic responses to the infused viruses, animals were treated with 8 mg/kg/day cyclosporin A (Calbiochem) and 2.5 mg/kg/day prednisone (Sigma) 2 days prior to adenovirus administration. The cyclosporin dose was doubled on the day of viral infusion and then changed to a daily maintenance dose of 4 mg/kg/day for 5 more days. Prednisone was discontinued on day 3 after viral infusion. Animals were infused with 1×10^{12} virions of either the AdCMV-G6Pase or AdCMV-β-Gal viruses in 200–250 μl of phosphate-buffered saline via a 22-gauge catheter inserted in the major tail vein. Cyclosporin levels were measured at different time points to ensure appropriate therapeutic levels (Cyclo-Trac I-125 RIA kit, Incstar Corp.), and plasma alkaline phosphatase and creatinine were measured as an index of cyclosporin-related liver or kidney damage, using a kit and methods provided by Sigma. Finally, possible adenovirus-mediated hepatotoxicity was monitored by daily measurement of plasma aspartate aminotransferase (Sigma Diagnostic kit). A normal rate of weight gain was found to be restored within 3 days of viral infusion (data not shown), consistent with previous findings (23).

Animals treated in the foregoing manner were subjected to one of the following experimental protocols. For protocol 1, animals were allowed to feed *ad libitum* for 7 days after viral infusion. During this 7-day interval, blood samples were collected on a daily basis for analysis of metabolic variables (see below). At the end of this period, animals were sacrificed for harvest of tissues. For protocol 2, animals were allowed to feed *ad libitum* for 5 days after viral infusion and then were subjected to an overnight fast (all food removed but water still available). Fasted animals were then subjected to an oral glucose tolerance test (OGTT), involving administration of 2 g/kg glucose via oral gavage. Blood samples were obtained immediately prior to the glucose gavage (0 time point) and at 30, 60, 90, and 120 min after the glucose load and used for assays of various metabolic parameters (see below). At the end of the 120-min glucose tolerance test, animals were sacrificed for tissue collection. For protocol 3, animals were allowed to feed *ad libitum* for 4 days and then fasted for 48 h. These animals were subjected to an OGTT identical to that described in protocol 2. At the conclusion of the OGTT, animals were provided with a measured amount of food (20 g) and allowed to refeed for an additional overnight period. At the conclusion of this refeeding period, blood samples were collected, and animals were sacrificed for tissue collection.

Assay of G6Pase Enzymatic Activity and Immunodetectable Protein in Liver Microsomes—Fresh liver samples (1 g) were homogenized in 50 mM Tris, pH 7.5, 0.25 M sucrose, 5 mM EDTA, and 1 μg/ml phenylmethylsulfonyl fluoride. G6Pase enzymatic activity was assayed in 100,000 × *g* microsomal fractions as described previously (19) using 40 μg of microsomal protein per assay (24). Levels of the G6Pase catalytic subunit protein were also measured by immunoblot analysis with a polyclonal antiserum raised against the catalytic subunit of glucose-6-phosphatase of rat (25), using previously described methods (19).

Metabolite and Hormone Assays—Blood samples were collected in tubes or syringes pre-rinsed with 0.1 M EDTA and centrifuged at 10,000 × *g* for 5 min. Plasma glucose concentrations were measured with a HemoCue glucose analyzer. Insulin radioimmunoassay was carried out with 25 μl of plasma, using the Linco rat insulin kit and rat insulin standards. Plasma triglyceride levels were determined with a Sigma triglyceride-GPO kit, and free fatty acids (FFA) were measured in fresh plasma with the Half-Micro test kit from Boehringer Mannheim. Liver glycogen levels were measured as described (26). To measure muscle triglyceride levels, 1 g of frozen gastrocnemius muscle was homogenized in 2 ml of buffer consisting of 18 mM Tris, pH 7.5, 300 mM mannitol, 50 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. After removal of a small aliquot of the homogenate for determination of total protein, 100 μl of the homogenate was mixed with 4 ml of chloroform/methanol mixture (2:1, v/v) for extraction of total lipids. After insoluble materials were filtered from the chloroform/methanol/lipid solution, 0.8 ml of water was added to separate the organic phase containing tissue lipid from the aqueous phase. A portion of the triglyceride-containing solution was dried under nitrogen gas, resuspended in 60 μl of *t*-butyl alcohol, and then mixed with 40 μl of Triton/methanol (2:1, v/v) to completely dissolve the lipid suspension, and an aliquot of this solution was used for triglyceride assay with the Sigma triglyceride-GPO kit.

RESULTS

Increased Expression of the G6Pase Catalytic Subunit in Liver of Zucker Diabetic Fatty (ZDF) Rats—Defective suppression of hepatic glucose output is a hallmark of human NIDDM. Like human NIDDM subjects, ZDF rats exhibit profound hyperglycemia, hyperinsulinemia, and hyperlipidemia, and impaired control of hepatic glucose production (27–31). The potential role of hepatic glucose-6-phosphatase expression in development of diabetes in ZDF rats has never been investigated, although increased expression of the mRNA encoding the G6Pase catalytic subunit in the islets of Langerhans of these animals has been reported (32). We therefore evaluated expression of the G6Pase catalytic subunit by immunoblot analysis and measurement of enzymatic activity in liver microsomes isolated from obese ZDF diabetic males, lean non-diabetic ZDF males of the same age (9 weeks), and weight-matched Wistar male rats. We observed a 2.4-fold increase in hepatic glucose-6-phosphatase enzymatic activity in ZDF diabetic animals compared with either control group, and this increase was accompanied by a similar increase in the expression of immunodetectable G6Pase catalytic subunit (Fig. 1). Glucose-6-phosphatase enzymatic activity was not detectable in skeletal muscle of ZDF diabetic males, ZDF lean controls, or Wistar rats, providing evidence that expression of G6Pase was not activated in other important homeostatic tissues in diabetic animals. These data suggest that up-regulation of hepatic G6Pase expression could contribute to development of the diabetic phenotype in ZDF rats and led us to investigate the specific metabolic impact of overexpression of the enzyme in liver of normal rats.

Effects of G6Pase Overexpression in Wistar Rats Fed Ad Libitum—Normal Wistar rats were infused with AdCMV-G6Pase or AdCMV-β-Gal and allowed access to food and water *ad libitum*. Seven days after viral infusion, blood samples were collected for measurement of circulating metabolites and hormone levels prior to sacrificing the animals for tissue collection. At this time point, circulating levels of aspartate aminotransferase, alkaline phosphatase, and creatinine were all within the normal range in both the AdCMV-G6Pase and AdCMV-β-Gal-treated animals, indicating no substantial liver or kidney

damage in response to viral infusion (data not shown). Liver microsome samples were used for analysis of levels of the G6Pase catalytic subunit by immunoblotting and measurement of enzymatic activity. A representative immunoblot showing the clear increase in hepatic expression of the G6Pase catalytic subunit protein in AdCMV-G6Pase compared with AdCMV-β-Gal-treated animals is shown in Fig. 2. This increase in protein corresponded to a 2.9-fold increase in G6Pase enzymatic activity measured in freshly isolated microsomes (Table I).

Previous studies in mouse have demonstrated that systemic administration of recombinant adenovirus results in gene transfer primarily to liver (22). To ensure that this was also the case in the current studies performed in rats, we measured glucose-6-phosphatase enzymatic activity in skeletal muscle, abdominal fat, and pancreas samples from the same AdCMV-G6Pase- and AdCMV-β-Gal-treated animals used for the hepatic measurements described above. We found no detectable activity in muscle or pancreas of either AdCMV-G6Pase- or AdCMV-β-Gal-treated animals. G6Pase activity was found at very low levels in abdominal fat but with no significant differences between the two experimental groups (0.107 ± 0.016 units/mg protein compared with 0.096 ± 0.015 units/mg in fat tissues of AdCMV-G6Pase- and AdCMV-β-Gal-infused animals, respectively, $n = 6$ for both groups). Based on these data, we conclude that the G6Pase gene was not targeted to these important extrahepatic homeostatic tissues by the adenovirus vector.

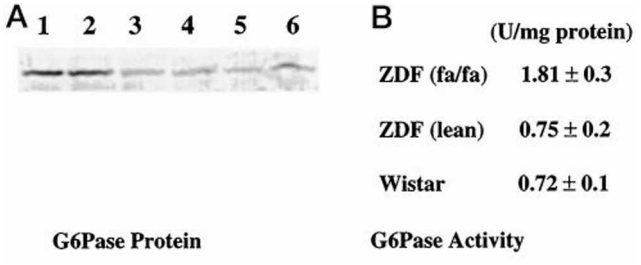

FIG. 1. **Expression of G6Pase in ZDF (fa/fa) rats and lean controls.** *A,* liver microsomes were prepared from 9-week-old (fa/fa) male ZDF diabetic rats (*lanes 1* and *2*), age-matched ZDF lean control animals (*lanes 3* and *4*), and normal Wistar rats, weight-matched to the ZDF diabetic males (*lanes 5* and *6*), and subjected to immunoblot analysis with an antibody recognizing the G6Pase catalytic subunit, as described under "Materials and Methods." *B*, G6Pase enzymatic activity was measured in liver microsome preparations from ZDF (fa/fa), ZDF lean, and normal Wistar rats, including samples represented in *A*. Data represent the mean ± S.E. for three independent determinations per group.

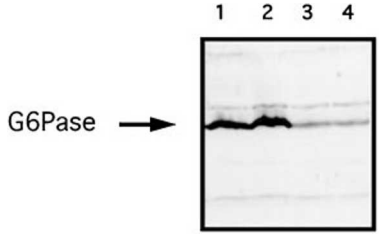

FIG. 2. **Immunoblot analysis of G6Pase catalytic subunit expression in liver of AdCMV-G6Pase-treated and control Wistar rats.** Rats were infused with AdCMV-G6Pase (*lanes 1* and *2*) or AdCMV-β-Gal (*lanes 3* and *4*) and allowed access to food *ad libitum* for 7 days prior to tissue collection ("Materials and Methods," see protocol 1). Each lane contains 40 μg of protein from liver microsome homogenates, blotted with an antibody against the catalytic subunit of G6Pase (25). Data are representative of results in all three protocols (see Tables I—III for enzymatic activity measurements for all animals in the study).

Table I provides a summary of a group of metabolic parameters assayed in the AdCMV-G6Pase and AdCMV-β-Gal animals fed *ad libitum*. Circulating glucose levels were raised by 14% and insulin levels by 47% in AdCMV-G6Pase-treated animals relative to controls. Despite the mild hyperglycemia and hyperinsulinemia, liver glycogen levels were reduced by 57% in AdCMV-G6Pase-treated animals. In contrast, lipid metabolism was regulated in a manner consistent with the elevated insulin levels. Circulating FFA and triglycerides (TG) were decreased by 37 and 29%, respectively, in AdCMV-G6Pase-treated animals (Table I), whereas triglyceride levels in isolated muscle samples were increased by 59% (Fig. 3).

Effects of G6Pase Overexpression in Fasted Rats Subjected to an Oral Glucose Tolerance Test—The increases in circulating glucose and insulin levels in AdCMV-G6Pase-treated rats fed *ad libitum* was suggestive of a state of glucose intolerance. To evaluate this possibility in more detail, animals were infused with AdCMV-G6Pase or AdCMV-β-Gal viruses, allowed free access to food for the ensuing 5 days, and were then fasted overnight. After the fast, both groups were subjected to an oral glucose tolerance test. At the end of this test, animals were sacrificed for tissue collection. AdCMV-G6Pase-infused animals had 2.3-fold more hepatic G6Pase activity than AdCMV-β-Gal-infused controls (Table II). As shown in Fig. 4, circulating glucose levels were not different in AdCMV-G6Pase-treated compared with AdCMV-β-Gal-treated animals after the overnight fast and prior to initiation of the OGTT (0 time point).

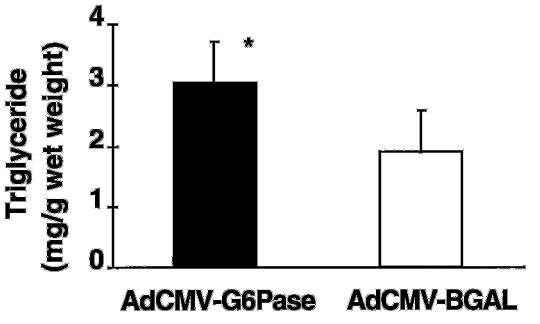

FIG. 3. **Muscle triglyceride levels in AdCMV-G6Pase- and AdCMV-β-Gal-infused animals.** Muscle triglyceride levels were measured as described under "Materials and Methods." Data represent the mean ± S.E. for six animals per condition (see Table I for other metabolic parameters for this same set of animals). The * indicates that muscle TG levels are elevated in AdCMV-G6Pase-infused animals relative to controls, at a level of significance of $p < 0.02$.

TABLE I

Metabolic parameters of rats fed ad libitum after infusion of AdCMV-G6Pase or AdCMV-β-Gal adenoviruses

Blood and liver samples were collected from rats fed ad libitum for 7 days after virus infusion, as described under "Materials and Methods," protocol 1. Results represent the mean ± S.E. for assays performed in duplicate on samples taken from the number of animals shown in parentheses in the left hand column.

Viral treatment	G6Pase activity	Plasma glucose	Plasma insulin	Plasma FFA	Plasma triglycerides	Liver glycogen
	units/mg protein	*mg/dl*	*ng/ml*	*mM*	*mg/dl*	*μg/mg protein*
AdCMV-G6Pase ($n = 6$)	1.47 ± 0.6	184 ± 12	2.2 ± 0.9	0.72 ± 0.3	124 ± 29	240 ± 55
AdCMV-β-Gal ($n = 6$)	0.51 ± 0.1	161 ± 21	1.5 ± 0.7	1.14 ± 0.5	174 ± 62	563 ± 167
Statistical significance	$p < 0.01$	$p < 0.05$	$p < 0.03$	$p < 0.05$	$p < 0.04$	$p < 0.005$

TABLE II

Metabolic parameters in AdCMV-G6Pase and AdCMV-β-GAL-infused rats after an overnight fast and administration of an oral glucose tolerance test

Blood and liver samples were collected as described under "Materials and Methods," protocol 2. Results represent the mean ± S.E. for assays performed in duplicate on samples taken from the number of animals shown in parentheses in the left-hand column.

Viral treatment	G6Pase activity	Plasma glucose	Plasma insulin	Plasma FFA	Plasma triglycerides	Liver glycogen
	units/mg protein	*mg/dl*	*ng/ml*	*mM*	*mg/dl*	*μg/mg protein*
AdCMV-G6Pase ($n = 5$)	1.50 ± 0.3	229 ± 12	0.93 ± 0.4	1.9 ± 1.0	61.7 ± 6.6	235 ± 70
AdCMV-β-Gal ($n = 5$)	0.66 ± 0.2	180 ± 21	0.66 ± 0.1	2.8 ± 0.8	80.4 ± 11.7	523 ± 146
Statistical significance	$p < 0.002$	$p < 0.01$	$p < 0.05$	$p < 0.05$	$p < 0.02$	$p < 0.005$

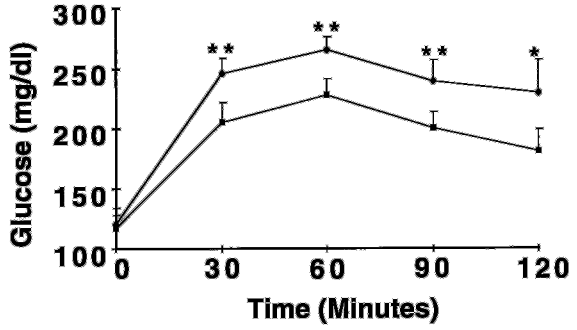

FIG. 4. **Oral glucose tolerance test in AdCMV-G6Pase- and AdCMV-β-Gal-infused animals after an overnight fast.** Animals were treated as indicated under "Materials and Methods," protocol 2. Data for AdCMV-G6Pase-treated animals (*black line, filled diamonds*) and AdCMV-β-Gal-treated controls (*gray line, filled squares*) are shown and represent the mean ± S.E. for five animals per group. Glucose levels were significantly higher in the AdCMV-G6Pase-treated rats at levels of significance indicated by the following symbols: *, $p < 0.01$; ** $p < 0.001$.

However, AdCMV-G6Pase-treated animals had significantly increased circulating glucose levels relative to control animals at all time points evaluated after administration of the oral glucose bolus, with a range of increase between 17 and 27%. Other serum parameters were measured only at the 120-min time point after oral glucose. At this time, plasma glucose and insulin levels were increased by 27 and 41%, respectively, in AdCMV-G6Pase- *versus* AdCMV-β-Gal-infused rats, whereas FFA and TG were decreased by 32 and 24%, respectively, similar to findings in animals fed *ad libitum*. Liver glycogen was decreased by 55% in AdCMV-G6Pase-infused animals, indicating that G6Pase overexpression causes a lowering of these stores in both animals fed *ad libitum* and in animals synthesizing glycogen in response to glucose infusion after a fast (Tables I and II).

Effects of G6Pase Overexpression on Fasted/Refed Rats—In animals fasted overnight, glycogenolysis is likely to make a significant contribution to hepatic glucose production, thus possibly obscuring the full impact of G6Pase overexpression during OGTT. To investigate this more fully, we evaluated the ability of AdCMV-G6Pase overexpressing animals to recover from a more extended period of fasting, and we also evaluated metabolic parameters after refeeding of the fasted animals. Rats were infused with AdCMV-G6Pase or AdCMV-β-Gal and allowed to recover for 4 days with *ad libitum* access to food. They were then fasted for 48 h, subjected to OGTT, and allowed to refeed on 20 g of chow/day for an overnight period. The amount of food consumed and the measured weight gain was the same for the AdCMV-β-Gal- and AdCMV-G6Pase-infused animals over the final day of the protocol (data not shown). At the end of the complete regimen, liver samples were collected and used immediately for assay of G6Pase enzymatic activity. G6Pase activity was increased by 67% in AdCMV-G6Pase-treated animals compared with AdCMV-β-Gal-treated controls (Table III). AdCMV-G6Pase-treated animals had similarly elevated levels of glucose during OGTT after a 48-h fast as they did after an overnight fast, whereas AdCMV-β-Gal-treated controls had a smaller increase in circulating glucose levels after a 48-h fast, indicating that the difference between groups was more apparent in the 48-h fasted animals (Fig. 5). The range of differences in blood glucose levels between AdCMV-G6Pase- and AdCMV-β-Gal-treated 48-h fasted animals during the glucose tolerance test was 23–52%. In blood samples taken after refeeding of the 48-h fasted animals, glucose and insulin levels were increased by 24 and 91%, respectively, whereas plasma FFA and triglycerides were decreased by 57 and 33%, respectively, in AdCMV-G6Pase-infused animals relative to controls (Table III). Glycogen levels were reduced by 53% in AdCMV-G6Pase-treated animals, similar to the decreases observed in protocols 1 and 2. However, absolute levels of glycogen were approximately 4 times higher in 48-h fasted and refed animals than in overnight fasted or animals fed *ad libitum* (compare Tables I–III). An "overshoot" of glycogen synthesis in animals refed after prolonged fasting has been previously described (33) and may explain the difference between groups in this study. Nevertheless, significant perturbation of a variety of metabolic parameters remains evident in G6Pase overexpressing compared with control rats even after allowing animals to refeed following a fast.

DISCUSSION

A failure to suppress hepatic glucose output in the postprandial state is a hallmark metabolic lesion of NIDDM. The molecular basis of this abnormality has never been clearly established. Based on studies of partially pancreatectomized rats, an experimental model of NIDDM, Rossetti and colleagues (12) have suggested that a reduction in the activity of the hepatic glucose phosphorylating enzyme glucokinase, or an increase in the activity of hepatic G6Pase, or a combination of both changes could result in reduced capacity for glucose utilization and storage in liver. In this study, we provide the first evidence that both hepatic expression of the catalytic subunit of G6Pase and glucose-6-phosphatase enzymatic activity are significantly increased in a genetic model of obesity and NIDDM, the ZDF rat (fa/fa). However, this finding does not establish a causal link between G6Pase expression and the diabetic phenotype. Furthermore, linkage between mutations in the gene encoding the G6Pase catalytic subunit and human NIDDM have not yet been established. While the search for such genetic mutations continues, further insight into the potential role of G6Pase in the development of NIDDM may be gained by studies in which the enzyme is specifically overexpressed in liver. In the current study, we have used systemic infusion of recombinant adenovirus, a technique that provides near exclusive delivery of transgenes to liver (see Ref. 22 and this study), to investigate this issue.

Our experiments establish that hepatic overexpression of the catalytic subunit of G6Pase, even to a relatively modest extent (1.6–3-fold increases in enzyme activity), is sufficient to cause significant perturbation of fuel homeostasis. The pattern of metabolic abnormalities was similar in all three experimental

TABLE III

Metabolic parameters in AdCMV-G6Pase and AdCMV-β-Gal-infused rats after a 48-h fast, administration of an oral glucose tolerance test, and an overnight period of re-feeding

Blood and liver samples were collected as described under "Materials and Methods," protocol 3. Results represent the mean ± S.E. for assays performed in duplicate on samples taken from the number of animals shown in parentheses in the left-hand column

Viral treatment	G6Pase activity	Plasma glucose	Plasma insulin	Plasma FFA	Plasma triglycerides	Liver glycogen
	units/mg protein	*mg/dl*	*ng/ml*	*mM*	*mg/dl*	*μg/mg protein*
AdCMV-G6Pase (n = 11)	0.94 ± 0.2	183 ± 19	2.1 ± 1.1	0.58 ± 0.2	59.2 ± 17.1	948 ± 289
AdCMV-β-Gal (n = 11)	0.56 ± 0.1	148 ± 17	1.1 ± 1.0	1.35 ± 0.4	87.7 ± 20.1	2009 ± 208
Statistical significance	$p < 0.002$	$p < 0.01$	$p < 0.03$	$p < 0.05$	$p < 0.05$	$p < 0.001$

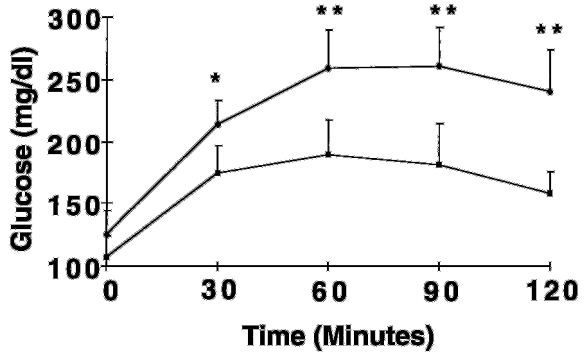

FIG. 5. **Oral glucose tolerance test in AdCMV-G6Pase- and AdCMV-β-Gal-infused animals after a 48-h fast.** Animals were treated as described under "Materials and Methods," protocol 3. Data for AdCMV-G6Pase-treated animals (*black line, filled diamonds*) and AdCMV-β-Gal-treated controls (*gray line, filled squares*) are shown and represent the mean ± S.E. for 11 animals per group. Glucose levels were significantly higher in the AdCMV-G6Pase-treated rats at levels of significance indicated by the following symbols: *, $p < 0.01$; ** $p < 0.001$.

protocols (*ad libitum* fed, overnight fasted + OGTT, and 48-h fasted + OGTT + refeeding). Specifically, AdCMV-G6Pase-treated animals exhibited glucose intolerance and mild hyperinsulinemia but were hypolipemic, as reflected in lowered levels of circulating FFA and TG. Levels of TG in muscle were also assessed for the group fed *ad libitum* and were found to be elevated in animals infused with AdCMV-G6Pase. The most readily apparent explanation for the circulating hypolipemia and tissue lipid overstorage is that the elevated insulin levels secondary to the glucose intolerance in the AdCMV-G6Pase-treated group caused suppression of lipolysis and activation of fatty acid esterification in peripheral tissues. In addition, G6Pase overexpression may have caused reduced glucose utilization in liver, resulting in suppression of *de novo* lipogenesis. Although glucose utilization was not measured directly in this study, glucose usage and lactate production were previously shown to be decreased in AdCMV-G6Pase-treated hepatocytes or INS-1 cells (19, 20). The finding of mild hyperglycemia or glucose intolerance in all three of the current experimental protocols is also consistent with enhanced hepatic glucose production and reduced hepatic glucose clearance in G6Pase overexpressing animals. Further studies will be required to delineate the relative contributions made by peripheral *versus* hepatic metabolism to perturbed lipid homeostasis in AdCMV-G6Pase-treated animals.

Overexpression of the G6Pase catalytic subunit in liver of normal rats was not sufficient to cause frank diabetes, since blood glucose levels were identical in overnight fasted AdCMV-G6Pase-treated and control animals (see 0 time point, Fig. 3). However, overexpression of the enzyme clearly caused a condition of glucose intolerance, as indicated by the significant increase in circulating glucose levels in animals fed *ad libitum* and the abnormal glucose tolerance test after either an overnight or 48-h fast. Glucose intolerance appears to be exacerbated in animals fasted for longer periods, especially when considering that the extent of overexpression of G6Pase in the 48 fasted animals was somewhat smaller than in the overnight fasted group (increases of 67 *versus* 127%, respectively). One potential explanation for the enhanced glucose intolerance with more prolonged fasting may relate to glycogen metabolism. Hepatic glycogen levels were decreased to around 100 μg/mg protein in both AdCMV-G6Pase- and AdCMV-β-Gal-treated animals after an overnight fast and prior to the OGTT (data not shown). These values were reduced from 240 and 563 μg/mg glycogen in livers of *ad libitum* fed G6Pase overexpressing and control animals, respectively (Table I). Thus, control animals had a larger glycogen depot to contribute to hepatic glucose production during the first 24 h of fasting, which may have counterbalanced the increase in glucose 6-phosphate hydrolysis in G6Pase overexpressing animals, resulting in equal fasting glucose levels. At later time points, the metabolic impact of G6Pase overexpression becomes more evident due to the lower background of glucose production in the control animals. Consistent with this idea, prior to the OGTT, blood glucose levels in 48-h fasted AdCMV-G6Pase-treated rats tended to be higher than in controls (125 ± 19 *versus* 107 ± 17 mg/dl), although this difference did not quite achieve statistical significance ($p = 0.06$).

Despite the mild hyperglycemia and hyperinsulinemia, glycogen levels were decreased by 53–58% in the three groups of AdCMV-G6Pase-treated animals relative to controls (Tables I–III). Interestingly, liver glycogen levels are also reduced in humans with NIDDM (27). Our findings in intact rats are consistent with our earlier study in isolated hepatocytes, in which a 50% decrease in glycogen accumulation was noted in AdCMV-G6Pase-treated cells compared with controls (20).

Some of the alterations in fuel metabolism in response to G6Pase overexpression reported in this study are observed in NIDDM, including glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content, and increased peripheral lipid storage. Other features, most notably hypolipidemia, are not commonly found in NIDDM. These findings raise the question of whether up-regulation of G6Pase can make a significant contribution to the development of the disease. We propose two models that represent opposite extremes for explaining how this could occur. The first assumes that increased expression of G6Pase is the primary genetic lesion associated with the disease. In this scenario, a gradual rise of G6Pase overexpression will result in development of glucose intolerance and hyperinsulinemia, resulting in turn in increased lipid storage in peripheral tissues, as demonstrated in this study. Peripheral overstorage of fat is strongly correlated with development of insulin resistance (reviewed in Ref. 34). If a causal role for lipid overstorage is correct, lipid-induced insulin resistance will cause the hormone to lose its capacity to inhibit peripheral lipolysis, resulting in a gradual rise in circulating FFA and TG. It might also be anticipated that peripheral glucose disposal would be decreased as insulin resistance develops. This model predicts further that overexpression of G6Pase in the liver for

periods of weeks to months (as opposed to the 7-day time course of the current study) might result in gradual development of hyperlipidemia and hyperglycemia, complementing hyperinsulinemia, and reduced hepatic glycogen levels to produce the entire syndrome of NIDDM. This hypothesis can be tested directly in future experiments.

The second model assumes that there are no genetic abnormalities in the G6Pase gene or its regulatory factors in individuals with NIDDM. In this scenario, overexpression of G6Pase in liver could instead be secondary to metabolic variations that occur in response to genetic lesions in insulin signaling or β-cell function. For example, expression of the G6Pase catalytic subunit has been shown to be increased by elevations in FFA or glucose in both *in vivo* and *in vitro* rodent experiments (13–18). Thus, G6Pase overexpression may be induced by rising FFA levels and glucose intolerance at an intermediate stage of NIDDM development. Induction of the gene could then contribute to failure of normal suppression of hepatic glucose production in the fed state, resulting in exacerbation of glucose intolerance and reduced glycogen storage. If up-regulation of G6Pase occurs at a fairly late stage of development of NIDDM, insulin resistance and β-cell failure may already be present, thus negating the role of hyperinsulinemia.

It should be noted that intermediate models, in which G6Pase interacts in different ways with other candidate genes that predispose to insulin resistance or β-cell failure, can also be contemplated. For example, it was initially surprising that mice homozygous for knockout of insulin receptor substrate 1 were found to be modestly insulin-resistant but with no abject hyperglycemia (35, 36). It has since become apparent that breeding of mice that are heterozygous for knockout of insulin receptor substrate 1 with mice heterozygous for knockout of other candidate genes such as β-cell glucokinase or the insulin receptor can result in frank diabetes (37, 38). Adenovirus-mediated gene delivery to liver of these various transgenic strains may provide new insights into the potential interaction of G6Pase overexpression with other genetic factors predisposing to NIDDM.

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