SPECIFIC REDUCTION OF HEPATIC GLUCOSE-6-PHOSPHATE TRANSPORTER-1 AMELIORATES DIABETES WHILE AVOIDING COMPLICATIONS OF GLYCOCEN STORAGE DISEASE

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Running Title: Reduction in G6PT1 ameliorates diabetes

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D-Glucose-6-phosphatase (G6Pase) is a key regulator of endogenous glucose production, and its inhibition may improve glucose control in type 2 diabetes. Herein, 2′-O-(2-methoxy)-ethyl-modified phosphorothioate antisense oligonucleotides (ASO) specific to the glucose-6-phosphate transporter-1 (G6PT1) enabled reduction of hepatic G6Pase activity in diabetic ob/ob mice. Treatment with G6PT1 ASOs decreased G6PT1 expression, reduced G6PT1 activity, blunted glucagon-stimulated glucose production, and lowered plasma glucose concentration in a dose-dependent manner. In contrast to G6PT1 KO mice and patients with glycogen storage disease, excess hepatic and renal glycogen accumulation, hyperlipidemia, neutropenia, and elevations in plasma lactate and uric acid did not occur. In addition, hypoglycemia was not observed in animals during extended periods of fasting, and the ability of G6PT1 ASO-treated mice to recover from an exogenous insulin challenge was not impaired. Together, these studies demonstrate that effective glucose lowering by G6PT1 inhibitors can be achieved without adversely affecting carbohydrate and lipid metabolism.

Hepatic control of glucose homeostasis is achieved by coordinating signaling pathways that regulate glycogen synthesis, glycogenolysis, and gluconeogenesis (1-3). During nutrient intake, D-glucose is taken up from the circulation into hepatocytes by the GLUT2 transporter. Glucokinase (GCK)/hexokinase 4 phosphorylates D-glucose at the sixth carbon position yielding D-glucose-6-phosphate (G6P) which is converted into G1P for glycogen synthesis and to fructose-6-phosphate for lipid and amino acid synthesis and energy metabolism. In the post-absorptive state, glycogen phosphorylase catalyzes the release of glucose from chains of glycogen, and as this supply is depleted, de novo glucose is synthesized from lactate, amino acids, and glycerol via the process of gluconeogenesis. The end product of both glycogenolysis and gluconeogenesis is G6P, which must be hydrolyzed by D-glucose-6-phosphatase (G6Pase) to yield D-glucose that can be released into the bloodstream. Together, the combined net flux through GCK and G6Pase determine the contribution of the liver to whole body glucose homeostasis.

G6Pase is a multi-component enzyme anchored in the ER that is comprised of membrane spanning catalytic (G6PC-α and/or G6PC-β) (4-8) and transport subunits (G6PT1, T2, T3) (9-11). The G6Pase substrate transport model proposes that the active site of G6PC is located on the cisternal surface of the ER lumen (12-14). The T1 transporter shuttles intracellular G6P across the ER membrane into the lumen where it is hydrolyzed, and the T2 and T3 transporters mediate export of inorganic phosphate (P) and glucose, respectively, back to the cytoplasm (13,15,16).

Inability to suppress hepatic glucose production due to insulin deficiency or resistance is a key defect in diabetes, and thus, attempts to develop molecules that inhibit glycogenolytic or gluconeogenic enzymes have been widely pursued...
as new treatments (17-19). In addition to the liver, the kidneys and intestine are capable of endogenous glucose production, although hepatic glucose production is quantitatively more important and regulatory control of the liver by hormones such as insulin and glucagon is better understood. As with hepatic glucose production, G6Pase is also required for glucose release by kidney and intestine. Because G6Pase is the obligate terminal control point for release of glucose generated by either glycogenolysis or gluconeogenesis, it is an attractive target for inhibiting hepatic glucose production. Unfortunately, the complete loss of a functioning G6Pase system leads to severe metabolic abnormalities. Glycogen storage disease type 1a (GSD-1a) is caused by a deficiency of G6PC-α (4), and GSD type 1b (GSD-1b) results from mutations in G6PT1 (9,20). G6PC-α and G6PT1 are co-dependent (15), and inactivating mutations in either gene lead to multiple physiologic disturbances, including hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidosis (reviewed in 21,22). Recently, however, compelling evidence indicates that limited replenishment of G6Pase activity in rodent models of GSD via adenovirus-mediated gene therapy can normalize plasma glucose, cholesterol, and triglyceride concentrations and dramatically reduce liver and kidney enlargement and glycogen deposition in these organs (23-25).

Thus, due to its key regulatory role in hepatic glucose production and because only limited G6Pase activity is needed to avoid complications of GSD, we chose to test the hypothesis that reducing activity of the G6Pase system would improve hyperglycemia in diabetes using 2'-O-(2-methoxy)-ethyl-modified phosphorothioate antisense oligonucleotides (ASO) specific to the G6PT1. The use of this chemical platform is advantageous because active molecules can be quickly identified, synthesized, and tested directly in primary hepatocytes and rodent models of type 2 diabetes (26-28). In addition, the pharmacokinetic properties of ASOs enable performing studies evaluating the long-term inhibition of targeted genes (29). The studies presented here demonstrate that chronically decreasing hepatic G6Pase activity in diabetic mice effectively lowers their blood glucose concentrations. Importantly, this occurs without excessive hepatic glycogen accumulation, hyperlipidemia, hyperuricemia, lactic acidosis, or hypoglycemia.

Experimental Procedures

Design and identification of the G6PT1 ASO inhibitor - A total of 175 ASOs targeting mouse G6PT1 sequences were designed as 20 base full-phosphorothioate chimeric 2'-O-(2-methoxy)-ethyl modified ASOs. These molecules represent a class of 20-base chimeric ASOs in which a RNase H-sensitive stretch of ten 2'-deoxy residues is flanked on both sides with a stretch of five 2'-O-(2-methoxy)-ethyl modifications. This modification increases mRNA binding affinity and confers nuclease resistance, while the chimeric design supports an RNase H terminating mechanism (30). Lead ASOs were screened in primary mouse hepatocytes as previously described (28). Following in vitro characterization of lead molecules from the initial screens, G6PT1 ASO ISIS 149008 (hybridizes to bases 1860-1879 of mouse G6PT1 sequence NM 008063.1) was identified as the most potent G6PT1 ASO. The control ASO, ISIS 141923, used in these studies is of the same chemistry, design, and length as the active G6PT1 ASO. It is a generic chemistry control that does not have perfect complementarity to any gene in public databases (sequence 5'-CCCTCCCCGAAAGGTTCCCTCC-3').

Animal care and treatments - C57Bl/6OlaHsd-Lepob (ob/ob) male mice and lean (C57Bl/6 or db1/+) male mice were purchased from Harlan (Indianapolis, IN). Animals were acclimated for one week prior to study initiation. Mice were housed five per cage in polycarbonate cages with filter tops and maintained on a 12:12 h light-dark cycle (lights on at 6:00 AM) at 21°C. All animals received de-ionized water ad libitum. Lean mice received Purina Diet 5008, while ob/ob mice received Purina Diet 5015 ad libitum. All animals were maintained in accordance with the Institutional Animal Use and Care Committee of Eli Lilly and Company and the NIH “Guide for the Use and Care of Laboratory Animals”. G6PT1 and control ASOs were prepared in normal saline, and the solutions were sterilized through a 0.2 μm filter. Animals were dosed with ASO solutions twice per week (separated by 3.5 days) via
subcutaneous injection. Before the initiation of each study and periodically during the study, blood was collected by tail clip without anesthesia into EDTA plasma tubes. Food intake and body weights were measured weekly.

Metabolite and hematology measurements - Plasma concentrations of glucose, triglycerides, uric acid, lactate, AST, and ALT were determined using a Hitachi 912 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). For determination of liver triglycerides, 20% liver homogenates were prepared in deionized water using lysing matrix D tubes (Qbiogene Inc./Bio101 Systems, Carlsbad, CA). Triglycerides in liver homogenates were assayed enzymatically using the Hitachi 912 triglyceride assay. In addition, plasma levels of insulin and G-CSF were determined by Luminex immunoassay (Linco Diagnostic Services, St. Charles, MO). For measurement of hematologic parameters, whole blood samples in EDTA anticoagulant were analyzed with an ADVIA 120 (Bayer HealthCare LLC, Tarrytown, NY). Blood cell morphology was assessed by light microscopy using blood smears stained with an ADVIA S60 (Bayer HealthCare LLC).

Histology and electron microscopy - For light microscopic assessment, liver and sternal bone marrow were collected into 10% buffered neutral formalin at necropsy, processed routinely, embedded in paraffin, sectioned, and stained with H&E and/or PAS +/- diastase digestion. For electron microscopic examination, small tissue fragments of liver were collected into modified Karnovsky’s fixative at necropsy, post-fixed in osmium tetroxide and potassium ferrocyanide, processed routinely, embedded in epoxy resin blocks, and sectioned. Images were obtained from sections stained with uranyl acetate and lead citrate and/or from unstained sections with a Gatan Multiscan camera (Gatan Inc., Pleasanton, CA).

Liver slice and glycogen assay methods - Glucose output from liver slices and liver glycogen assays were performed as previously described (28). For kidney glycogen determination, one kidney from each animal was homogenized in 1 ml of 3M perchloric acid on ice. Samples were vortexed briefly, and 50 µl of extract was added to 500 µl of amyloglucosidase solution (50 mM Na Acetate + 0.02% BSA, pH 5.5 and 50 µg/ml amyloglucosidase, pH 5.5) to enzymatically cleave glycogen to glucose in an overnight incubation at room temperature. Glucose was measured in the supernatant by analyzing NADPH fluorescence after hexokinase/glucose-6-phosphate dehydrogenase reaction using standard methods, and glycogen content was reported as µmol glucose/mg kidney tissue.

Preparation of hepatic and renal microsomes - Microsomes from non-fasted mouse liver and kidney were isolated similar to the method of Williams et al. (31) using differential centrifugation. Tissues were homogenized at 1:1 w/v in buffered sucrose (50 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4) containing Complete Protease Inhibitor Tablets (Roche Diagnostics) using a teflon/glass, motorized Potter-Elvehjem tissue grinder. The homogenate was brought to 20% v/v with additional homogenization buffer and centrifuged 3,000 x g at 4 °C for 10 min. The supernatant was further centrifuged 18,000 x g at 4 °C for 15 min. The resulting supernatant retained the microsomal fraction and was placed in thick-wall polycarbonate tubes for ultracentrifugation at 100,000 x g at 4 °C for 60 min. The supernatant was removed, and the microsomal pellet was rinsed with storage buffer (5 mM HEPES, pH 7.3 with Complete Protease Inhibitor Tablets) to clear excess lipids. The microsomal pellet was resuspended with four strokes of a manual, Potter-Elvehjem mortar and pestle. Protein concentrations were determined using the modified Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA). Microsomes were either stored at -80 °C or directly tested in enzyme assays.

Measurement of G6PC and G6PT1 activity in microsomal preparations - G6Pase activity was assessed by the detection of P1, resulting from the hydrolysis of D-mannose-6-phosphate (M6P) or G6P (32,33). The latency of microsomal preparations was determined by assaying M6P hydrolysis in intact versus detergent-disrupted microsomes. G6Pase activity of intact microsomes was corrected for the portion of activity resulting from the disrupted vesicles within untreated preparations (13,32,34). Disodium G6P and disodium M6P salts were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Microsomes were either stored at -80 °C or disrupted by 0.2% sodium deoxycholate treatment for 20 min. Sixty mg of protein for intact microsomes and 6 mg of...
disrupted microsomes were assayed for G6Pase activity in 100 µl reactions at 30 °C in 50 mM sodium cacodylate, pH 6.5, 2 mM EDTA, and 5 mM substrate. After 15 min, reactions were terminated, and Pi was quantified by adding 900 µl of detection reagent containing six parts 0.42% ammonium molybdate tetrahydrate in 1 N H2SO4, two parts 2% SDS, and one part 1% ascorbic acid (33). The color was developed for 25 min at 45 °C, and absorbance was measured at 820 nm. External standards were generated by serial dilution of potassium phosphate monobasic, and unknowns were interpolated from the standard curve.

**RNA isolation, reverse transcription, and real-time quantitative RT-PCR** - RNA isolation and reverse transcription was performed as previously described (28). Real-time quantitative RT-PCR was performed using the 5' fluorogenic nuclease assay and a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) to determine the relative abundance of assayed mRNAs. Samples were normalized using Ribogreen (Molecular Probes, Inc., Eugene, OR) or the abundance of 36B4 mRNA. The 5' terminus of fluorogenic probes was labeled with FAM (6-carboxyfluorescein) or VIC. The 3' terminus contained the quenching dye TAMRA (6-carboxytetramethylrhodamine). Primers and probes were synthesized by Biosearch Technologies, Inc. (Novato, CA) or Applied Biosystems. Primer and probe sequences were as follows: mouse G6PT1 forward primer 5'-GAAGGCGAGCGTCTCTGTAT-3', mouse G6PT1 reverse primer 5'-CCATCCCAGCATCATGAG-3', mouse G6PT1 Taqman probe 5'-AACCCTCGCAGCATGTGAG-3'; mouse 36B4 forward primer 5'-GGCCCGAGAAGACCTCCTT-3', mouse 36B4 reverse primer 5'-TCAATGGTGCCTCTGGAGATT-3', mouse 36B4 Taqman probe 5'-CCAGGCTTGGGATCATCACCACG-3'. Primers and probes were also obtained as Applied Biosystems (http://www.appliedbiosystems.com/) Taqman Gene Expression Assays: G6PC-α (Mm00839363_m1), G6PC-β (Mm00616234_m1), G6PT1 (Mm00484574_m1), SREBP1 (Mm00550338_m1), SREBP2 (Mm01306294_m1), ACC1 (Mm01304257_m1), FAS (Mm00662319_m1), and PEPCK (Mm00440636_m1). PCR reactions were run in triplicate 20 µl reactions that contained Universal PCR Master Mix (Applied Biosystems), 4 pmols of each forward and reverse primer, 3 pmols of probe, and cDNA. Two-step PCR cycling was carried out as follows: 50 °C 2 min for 1 cycle, 95 °C 10 min for 1 cycle, and 95 °C 15 s, 60 °C 1 min for 40 cycles.

**Statistics** - Data are represented as mean ± SEM and were compared using the Student’s t-test. Repeated measures analysis of variance was used to assess the statistical significance between time courses. The Tukey method was used to adjust P-values for multiple comparisons. The null hypothesis was rejected at P<0.05.

**RESULTS**

In vitro screens identified the G6PT1 ASO (ISIS 149008), which decreased levels of G6PT1 mRNA in primary mouse hepatocytes in a dose-dependent manner (Fig. 1A). To test the hypothesis that lowering G6PT1 would decrease hepatic G6Pase activity and lower plasma glucose concentration in diabetes, 7-8 week-old ob/ob mice were dosed via subcutaneous injection two times per week with either G6PT1 ASO or a control ASO (ISIS 141923) for 4 weeks. Hyperglycemia continued to worsen over time in control ASO-treated animals, however, the concentration of plasma glucose decreased in G6PT1 ASO-treated ob/ob mice almost into the normal range (Fig. 1B). A similar effect on glucose lowering was observed in diabetic male db/db mice (data not shown). In the hyperinsulinemic ob/ob mice, improved glycemic control by G6PT1 ASO treatment resulted in reduced plasma insulin levels (11.7 ± 2.1 ng/ml in control ASO-treated ob/ob mice vs. 6.3 ± 1.3 ng/ml in G6PT1 ASO-treated ob/ob mice, P < 0.05). At the end of the 4-week treatment period, liver G6PT1 mRNA was reduced by 80–90% (Fig. 1B). No difference in food consumption was observed between control and G6PT1 ASO-treated animals (data not shown).

In dose ranging studies, plasma glucose concentration, the abundance of hepatic G6PT1 mRNA, and G6P hydrolysis in intact liver microsomes were all reduced in a dose-dependent manner by G6PT1 ASO therapy (Fig. 1C). The
reduction of G6P uptake in liver microsomes from G6PT1 ASO-treated mice was similar to results obtained in experiments evaluating G6P transport in microsomes treated with small molecular weight G6PT1 inhibitors (35-38). However, our results differ from those obtained with liver microsomes from patients with glycogen storage disease type 1b (GSD-1b) (39-41) and G6PT1 KO mice (42) where G6P transport was abolished. Importantly, though, the G6PT1 ASO hepatic microsome results mirror G6P uptake data from G6PT1 KO mice infused with adenoviral vector-containing G6PT1 where partial G6PT1 replenishment enabled some G6P transport (25). Thus, although reducing G6PT1 lowered blood glucose levels in diabetic rodents, maintaining some capacity to hydrolyze G6P is likely important because severe hypoglycemia and metabolic acidosis occur in animals and humans with GSD caused by the complete loss of a functioning G6Pase system (reviewed in 21,22).

The decrease in G6P transport in liver microsomes from G6PT1 ASO-treated mice (Fig. 1C) was consistent with a decrease in the rates of both basal and glucagon-stimulated glucose production from liver slices (Fig. 2A). A similar correlation has previously been observed in studies with G6PT1 inhibitors assessing glucose output from primary hepatocytes (36) and liver perfusion systems (35). Attempts to perform liver slice experiments in the diabetic models were unsuccessful due to technical difficulties isolating consistent cores from severely steatotic livers.

Although glucose output was significantly blunted, G6PT1 ASO treatment did not result in excessive accumulation of liver glycogen (Table 1). Consistent with the proposed mechanism of action, the decrease in hepatic glycogen content during a 16 h fast was blunted in animals receiving G6PT1 ASO treatment (Table 1). To further assess hepatic glycogen metabolism, non-diabetic mice were treated for 6 weeks and then challenged with extended periods of fasting. Again, depletion of liver glycogen was blunted in G6PT1 ASO-treated mice compared with controls at 6 h of fasting. However, hepatic glycogen content was indistinguishable between the groups at the 12 and 24 h time points (Fig. 2B).

To further investigate results from the biochemical assessment of hepatic glycogen content, liver tissues from fasted and fed ob/ob mice administered control or G6PT1 ASOs were collected and examined microscopically (Fig. 3). Compared to fasted control mice, periportal hepatocytes from fasted G6PT1 ASO-treated mice had mildly increased amounts of cytoplasmic vacuolation, consistent with glycogen (Fig. 3A, 3B). In contrast, fed G6PT1 ASO-treated mice did not have noticeable microscopic changes in hepatocellular glycogen (Fig. 3D). Further, by electron microscopy, there were no overall differences in the amount of glycogen (appearing lightly electron dense) or lipid in hepatocytes between control and G6PT1 ASO-treated animals (Fig. 3E, 3F). Because acute inhibition of G6PT1 activity by chlorogenic acid derivatives has been reported to increase de novo lipogenesis (35), we assessed the effect of G6PT1 ASOs on hepatic lipid metabolism. We demonstrated that plasma triglycerides were not affected by G6PT1 ASO treatment (Table 1). Similarly, liver triglycerides were not significantly changed by G6PT1 ASO treatment (Table 1). To further explore potential lipogenic effects of G6PT1 lowering, mRNA levels for SREBP1, SREBP2, ACC1, and FAS were examined; again, changes were not observed in any of these genes in livers from G6PT1 ASO-treated mice (Supplemental Fig. 1).

Due to the pivotal nature of the G6Pase system in controlling hepatic glucose production, we sought to assess the effects of G6PT1 ASO treatment on the ability of normal, non-diabetic mice to maintain euglycemia under conditions where endogenous glucose production is required. C57Bl/6 male mice were treated with control or G6PT1 ASOs for 6 weeks, thereby decreasing G6PT1 mRNA by 85% (Supplemental Fig. 2). These animals were subjected to carefully monitored periods of fasting up to 24 h (Fig. 4A). Plasma glucose concentrations were maintained after 6 and 12 h of fasting in G6PT1 ASO-treated animals despite the reduced G6PT1 levels. Only after 24 h of fasting did glucose levels fall below the control ASO group, and even then, glucose concentrations did not reach levels considered to be hypoglycemic. Unlike G6PT1 null mice (42), G6PT1 ASO-treated mice did not show hypoglycemic seizures at any time during these studies. Additionally, to assess potential changes in gluconeogenic enzymes before and after fasting in animals treated with the G6PT1 ASO, hepatic mRNA levels for G6PC-α, G6PC-β, and PEPCK...
were determined; an increase in G6PC-α was observed (see also Fig. 5C), but no changes were found for the other genes (Supplemental Fig. 2). To further evaluate the risk of hypoglycemia, we assessed the ability of G6PT1 ASO-treated animals to respond to a hypoglycemic challenge induced by an acute bolus of exogenous insulin (Fig. 4B). G6PT1 ASO-treated animals demonstrated a comparable fall in plasma glucose levels in response to an insulin injection, and the recovery from the fall in blood glucose concentration was equivalent if not more robust than animals treated with the control ASO.

Consistent with the G6Pase substrate transport model (12-14), detergent disruption of microsomes allows direct access of G6P to the catalytic site of G6PC. In the experiments presented here, G6PC activity was decreased in intact liver microsomes from G6PT1 ASO-treated mice (Fig. 1D, 5A); however, phosphohydrolase activity was increased upon detergent-disruption compared to controls (Fig. 5B). These findings are similar to those obtained in biochemical studies characterizing GSD-1b, where intact microsomes from GSD-1b patients were devoid of G6PC activity, however, upon microsomal disruption, higher than normal G6P hydrolysis was observed (39). Consistent with an increase in G6P hydrolytic activity, hepatic G6PC-α mRNA in G6PT1 ASO-treated mice was elevated compared with its abundance in liver tissue of control mice (Fig. 5C; Supplemental Fig. 2). Again, these results are in agreement with previous findings with G6PT1 inhibitors where hepatic G6PC-α expression was increased in animals treated with the chlorogenic acid derivatives, S3483 (43) and S4048 (44).

The kidney is also capable of contributing to whole body glucose production, however, G6PT1 ASO administration to ob/ob mice resulted in only a 45% decrease in kidney G6PT1 mRNA levels. This change in kidney G6PT1 transcripts did not lead to alterations in kidney wt (0.19 ± 0.02 g in control ASO-treated ob/ob mice vs. 0.18 ± 0.01 g in G6PT1 ASO-treated ob/ob mice) or renal glycogen content in the fed or fasted states (Table 1). The lack of adverse glycogen accumulation in the kidney by lowering renal G6PT1 by only 45% is not surprising because GSD is an autosomal recessive disorder, and metabolic complications do not manifest in heterozygote carriers. Further, an increase in phosphohydrolase activity was observed in detergent-disrupted kidney microsomes from G6PT1 ASO-treated mice (Fig. 5B). It is clear that the kidney possesses significant phosphohydrolase capability as both G6PC-α and -β are readily abundant (Fig. 5C, 5D). Analysis of the levels of G6PC-β did not reveal a change in its abundance upon G6PT1 ASO treatment. More sophisticated analyses similar to those using dual-isotope techniques (45) are needed to further assess renal glucose release in G6PT1 ASO-treated animals.

Upon determining that treatment with G6PT1 ASOs did not result in overstorage of liver or kidney glycogen, and concluding that treated animals were not more susceptible to experiencing adverse hypoglycemia, we sought to evaluate other metabolic disturbances that occur in GSD. For example, hyperuricemia and lactic acidosis result from both G6PC and G6PT1 mutations, and the loss of G6PT1 manifests an even more severe phenotype featuring neutropenia, neutrophil dysfunction, and an increased susceptibility to bacterial infection (reviewed in 21,22). Our results indicate that G6PT1 ASO administration did not result in the marked phenotypic changes that are characteristic of GSD-1b. A comparison of several metabolic parameters from animals treated for 4 weeks is presented in Table 2. In G6PT1 KO mice, lactate and uric acid concentrations are increased ~1.8-fold and ~2-fold, respectively (42). In our studies, plasma levels of lactate in lean animals treated with G6PT1 ASOs were unaltered compared to controls. In ob/ob mice undergoing G6PT1 ASO treatment, lactate concentrations were reduced ~2-fold in the fed state and elevated ~2.2-fold in the fasted state, although this increase did not exceed levels of circulating lactate found in fasted lean mice (Table 2). Uric acid concentrations were decreased in the fed state in ob/ob mice administered G6PT1 ASOs, although unchanged in the fasted state relative to controls (Table 2). Because immune dysfunction in GSD-1b primarily results from neutropenia and impaired leukocytes, a hematologic analysis was performed. Neither cell counts of neutrophils nor white blood cells were altered by G6PT1 ASO treatment for 4 weeks (Table 2). At necropsy, sternal bone marrow from ob/ob mice was collected for microscopic examination; no changes...
in bone marrow were found in comparisons of G6PT1 or control ASO-treated animals (data not shown). Finally, neutropenia in G6PT1-deficient mice is associated with a marked increase in the concentration of G-CSF, ranging from a ~1,700-fold increase in animals 2-3 weeks of age to 12-fold differences in 22-week old mice (42). Compared to controls, changes in G-CSF levels were not observed in mice receiving G6PT1 ASO treatment (Table 2). Overall, our experiments did not reveal the development of a GSD-like phenotype in mice administered G6PT1 ASOs, and thus, long-term studies to further evaluate the physiologic effects of this treatment are warranted.

DISCUSSION

Uncontrolled hepatic glucose output is a major contributor to elevated glucose levels in patients with type 2 diabetes, especially during fasting hyperglycemia (46). When considering the development of specific agents to treat excessive glucose production by the liver, biochemical and genetic data generated over the last several decades have provided compelling evidence to support targeting the G6Pase system. Indeed, disruption of glucose homeostasis in both humans and rodents possessing inactivating mutations in either $G6PC-\alpha$ or $G6PT1$ indicates that suppressing G6Pase activity would decrease hepatic glucose release (4,47). Strategically, developing inhibitors to this terminal control point may be advantageous because of uncertainty about whether to pursue molecules against glycogenolytic or gluconeogenic targets to treat excess hepatic glucose production in diabetes (48-50).

Although the initial characterization of small molecular weight antagonists targeting the G6Pase system provided supporting validation data to pursue G6Pase inhibitors for diabetes, effective glucose lowering in the absence of side effects related to GSD has yet to be reported. For example, chlorogenic acid and derivatives such as S3483 and S4048 possess reversible and competitive G6PT1 inhibitory activity in microsomal assays (35-38). Furthermore, intravenous administration of S3483 to fed rats prevented glucagon-induced hyperglycemia (51), and similar short-term experiments with S4048 demonstrated glucose lowering in diabetic $ob/ob$ mice (36). These studies support the prediction that G6PT1 inhibitors would possess anti-diabetic activity, however, the concurrent manifestation of adverse effects, including hypoglycemia, elevated levels of lactate, and altered carbohydrate and lipid metabolism, likely prevented further advancement of S4048 and similar compounds (52,53). Because the physiologic response of inhibiting activity of the G6PT1 subunit with these small molecule compounds appears to recapitulate the phenotype that manifests as a result from the loss of G6PT1 function, successful development of molecules possessing G6Pase inhibitory activity in all tissues appears unlikely.

In contrast to effects that result from the complete and ubiquitous loss of G6PT1, the studies presented here are the first to demonstrate that significant glucose lowering can be achieved by chronic but selective inhibition of hepatic G6PT1 activity without inducing adverse metabolic effects. G6PT1 ASOs lowered hepatic G6PT1 mRNA and G6P transport activity by 85% and 75%, respectively, resulting in decreased hepatic glucose production in ex vivo liver slice assays and reduced hyperglycemia in rodent models of diabetes. In 4-week studies, the decrease in hepatic G6PT1 nearly normalized blood glucose levels in diabetic $ob/ob$ and $db/db$ mice. Importantly, inhibition of G6Pase activity in these models was not associated with an excess accumulation of liver glycogen or elevated concentrations of serum triglycerides, cholesterol, lactate, or uric acid. In addition, diabetic mice treated with G6PT1 ASOs did not experience hypoglycemia when undergoing periods of fasting for 16 h. To more rigorously evaluate the risk of hypoglycemia, normal mice were treated for 6 weeks with G6PT1 ASOs and then subjected to either a 24 h fast or challenged with bolus insulin injections to assess their ability to recover from a hypoglycemic insult. Similar to results with diabetic animals, adverse hypoglycemia was not observed in normal animals undergoing G6PT1 ASO therapy. Further, control and G6PT1 ASO-treated mice were indistinguishable in their recovery response to the insulin challenge.

Several factors likely contribute to the intriguing observation that G6PT1 ASO treatment improves hyperglycemia without inducing a GSD-like phenotype. In contrast to patients and rodents with GSD-1b, G6P transport activity in the liver
was not completely abolished by G6PT1 ASO therapy. The 70-80% decrease of G6P hydrolysis in intact liver microsomes from G6PT1 ASO-treated ob/ob mice is similar to the level of hepatic G6Pase activity restored in G6PC-α KO animals by G6PC-α gene-bearing recombinant adenovirus treatment (23,24) and in G6PT1 KO mice by G6PT1 gene-bearing recombinant adenovirus treatment (25). These gene therapy approaches restored G6Pase activity in the liver to 19-38% of that of WT mice which was sufficient to normalize plasma glucose, cholesterol, triglycerides, and uric acid (23-25). Further, in the G6PT1 ASO-treated ob/ob mice, the reduction of G6PT1 activity resulted in a compensatory increase in hepatic G6PC-α levels and G6PC activity in detergent-disrupted liver microsomes. Additionally, G6PT1 mRNA was induced upon fasting by approximately 3-fold in both control and G6PT1 ASO-treated mice, although the absolute levels of G6PT1 mRNA was reduced by G6PT1 ASO-treatment. Finally, maintaining some capacity of the liver to mobilize glycogen for inter-prandial glucose homeostasis is supported by our results showing that hepatic glycogen levels were moderately reduced in G6PT1 ASO-treated animals during periods of fasting.

The biodistribution properties of the class of ASOs used in these studies result in high ASO concentrations in the liver and kidney (54). ASO distribution is quite broad in the liver leading to substantial target reduction in all cells (55). Utility of the ASO platform to achieve hepatic-targeted pharmacology has been demonstrated for diabetes (26-28), hepatitis C infection (56), and hyperlipidemia (57). Conversely, cell-type accumulation of ASOs in the kidney is heterogenous, which results in less overall target mRNA reduction in this tissue (54). It is likely advantageous that G6PT1 ASOs lower G6PT1 mRNA levels by only 45% in the kidney thereby avoiding alterations in kidney weight and renal glycogen content. Interestingly, although G6P transport was not lowered in kidney microsomes from G6PT1 ASO-treated mice, G6PC activity was significantly increased. This result suggests that kidneys in G6PT1 ASO-treated animals may compensate for the reduced ability of the liver to release glucose. We hypothesize that the kidney possesses this capacity because it expresses high amounts of both G6PC-α and the recently characterized G6PC-β (6,7). No other tissue contains high levels of both of these phosphohydrolases. Although not addressed in these studies, allosteric regulation by metabolites may also contribute to increased catalytic activity in the kidneys of treated animals (44). Additional work is needed to explore possible reciprocal changes in hepatic and renal glucose release in order to better define the contribution of the kidney to post-prandial glucose homeostasis in animals undergoing G6PT1 ASO treatment. Finally, the small intestine possesses the ability to contribute to whole body endogenous glucose production, and although G6PC-α is barely detectable, G6PC-β (7) and G6PT1 (10) mRNAs are very abundant. In G6PT1 ASO-treated mice, intestinal G6PT1 mRNA levels were unchanged compared to control animals (data not shown). For technical reasons, we did not evaluate G6P hydrolysis in intestinal microsomes from treated animals, however, the lack of G6PT1 mRNA lowering in this organ may allow it to also contribute glucose to the circulation, helping maintain euglycemia. This is unlike GSD-1b patients that possess a global loss of the transporter.

Finally, we do not completely understand the discrepancies in results presented here for the G6PT1 ASO with those reported from studies exploring chlorogenic acid G6PT1 inhibitors where adverse complications similar to those found in GSD were induced (52,53). Regardless, our findings do provide a new rationale to re-consider pursuing strategies to specifically target the hepatic G6Pase system for the treatment of hyperglycemia. Developing molecules such as the G6PT1 ASO that exhibit pharmacologic effects primarily in the liver may facilitate achieving better glucose control in type 2 diabetes. Also, similar to studies evaluating adenovirus-mediated gene therapy strategies for GSD, our results provide additional and complimentary evidence that GSD-related complications can be avoided if some hepatic G6Pase activity is available.
REFERENCES


**FOOTNOTES**

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Abbreviations used are: antisense oligonucleotide (ASO), glucokinase (GCK), glucose-6-phosphatase (G6Pase), glucose-6-phosphatase catalytic subunit alpha (G6PC-α), glucose-6-phosphatase catalytic subunit beta (G6PC-β), glucose-6-phosphate (G6P), glucose-6-phosphate transporter 1 (G6PT1), glucose-6-phosphate transporter 2 (G6PT2), glucose-6-phosphate transporter 3 (G6PT3), glycogen storage disease type 1a (GSD-1a), glycogen storage disease type 1b (GSD-1b), inorganic phosphate (Pi), mannose-6-phosphate (M6P).

**FIGURE LEGENDS**

**Fig. 1.** Reduction of G6PT1 mRNA, G6Pase phosphohydrolase activity, and glucose lowering in diabetic mice undergoing G6PT1 ASO treatment. *A,* G6PT1 ASO-mediated target reduction in primary hepatocytes. Mouse hepatocytes were treated with the indicated ASO concentration for 4 h. Following treatment, cells were cultured for an additional 16 h, total RNA was extracted, and G6PT1 mRNA levels were assessed by RT-PCR. Data are expressed as percent of saline-treated controls. *B* and *C,* Glucose-lowering and target reduction in diabetic mice undergoing G6PT1 ASO treatment. *B,* Plasma glucose and hepatic G6PT1 mRNA levels in 8-week-old male *ob*/*ob* mice treated twice per week (every 3.5 days) with
control ASO (squares) or G6PT1 ASO (triangles) for 4 weeks. Both ASOs were administered at 25 mg/kg (n = 5 mice per treatment group). Following the dosing period, liver tissues were harvested and G6PT1 mRNA levels determined by RT-PCR. G6PT1 mRNA was normalized to total RNA in the same samples using 36B4 mRNA. Data are the mean values ± SEM of five mice per treatment group. C, Dose-dependent glucose lowering, liver G6PT1 mRNA reduction, and hepatic microsomal G6Pase activity in ob/ob mice treated with G6PT1 or control ASOs. Plasma glucose and hepatic G6PT1 mRNA levels in 8-week-old male ob/ob mice treated with control ASO at 25 mg/kg (black bar) or G6PT1 ASO at 6.25 mg/kg (dark gray bar), 12.5 mg/kg (light gray bar), or 25 mg/kg (white bar) for 4 weeks. Following the dosing period, liver tissue was harvested and G6PT1 mRNA levels were determined by RT-PCR. G6PT1 mRNA was normalized to total RNA in the same samples using 36B4 mRNA. Data are the mean values ± SEM of five mice per treatment group (P < 0.05 using Student’s t test). G6Pase phosphohydrolase activity in intact microsomes from liver tissue of control and G6PT1 ASO-treated animals was determined by detecting Pi resulting from the hydrolysis of D-glucose-6-phosphate and is depicted in by the line graph (white boxes). In overall comparisons, glucose lowering, G6PT1 mRNA reduction, and microsomal G6Pase activity in animals treated with G6PT1 ASOs were significantly different compared individually with control ASO-treated animals (P < 0.05 adjusted using Tukey’s t test).

Fig. 2. Hepatic glucose metabolism in mice treated with G6PT1 ASOs. A, Ex vivo hepatic glucose production in C57Bl/6 mice. Glucose output was measured from liver slices obtained from normal mice treated with control (square) or G6PT1 (triangle) ASOs twice per week (every 3.5 days) for 6 weeks. Both basal and glucagon-stimulated rates of glucose production were lower in G6PT1 ASO-treated mice compared to controls. B, Glycogen breakdown during a period of 24 h fasting in C57Bl/6 mice treated with G6PT1 ASOs. Glycogen levels were measured in liver tissues from 12-week-old normal mice (n = 6 per treatment group), which had been treated twice per week (every 3.5 days) with 25 mg/kg control (square) or G6PT1 (triangle) ASOs.

Fig. 3. Photomicrographs of liver tissue from diabetic mice undergoing G6PT1 ASO treatment. H&E staining of representative liver sections from ob/ob mice administered control ASO (fasted, A; fed, C) or G6PT1 ASO (fasted, B; fed, D) twice per week (every 3.5 days) at 25 mg/kg for 4 weeks. Electron micrographs of representative liver sections from ob/ob mice administered control (E) or G6PT1 (F) ASO via the above described treatment regimen. L = lipid, N = nucleus of hepatocytes. Bar = 2 µm.

Fig. 4. Recovery from hypoglycemia in animals undergoing G6PT1 ASO treatment. A, Blood glucose levels during fasting periods of 6, 12, and 24 h in C57Bl/6 mice (n = 6 per treatment group) treated twice per week (every 3.5 days) with 25 mg/kg control (square) or G6PT1 (triangle) ASOs for six weeks. B, Insulin tolerance test in C57Bl/6 mice undergoing G6PT1 ASO treatment. Similar to the ASO treatment regimen described in A, mice were administered control (square) or G6PT1 (triangle) ASOs for 6 weeks and then challenged with bolus insulin injections to assess their ability to withstand a hypoglycemic insult.

Fig. 5. Liver and kidney G6PC phosphohydrolase activity and mRNA levels in G6PT1 ASO-treated diabetic mice. Glucose-6-phosphate (G6P) hydrolysis in intact (A) and detergent-disrupted (B) liver and kidney microsomes from 8-week-old male ob/ob mice treated twice per week (every 3.5 days) via subcutaneous injection of control (black bar) or G6PT1 ASO (white bar) for 4 weeks. Both ASOs were administered at 25 mg/kg. Following the dosing period, liver and kidney tissues were harvested for microsome isolation as described in the Methods. G6Pase activity in intact and detergent-disrupted microsomes from livers and kidneys of control and G6PT1 ASO-treated animals was determined by detecting Pi resulting from the hydrolysis of G6P. To assess changes in mRNA levels of both phosphohydrolases, total RNA was extracted from the same liver and kidney tissue samples and G6PC-α (C) and G6PC-β (D) mRNA levels were determined by RT-PCR. mRNA levels for both
phosphohydrolases were normalized to total RNA in the same samples using 36B4 mRNA. Data are the mean values ± SEM of five mice per treatment group (P < 0.05 using Student’s t test).

**Supplemental Fig. 1.** Assessment of lipogenic gene expression in livers from ob/ob mice undergoing G6PT1 ASO treatment. Eight-week-old male ob/ob mice (n = 5 per treatment group) were treated twice per week (every 3.5 days) with control ASO or G6PT1 ASO for 4 weeks. Both ASOs were administered at 25 mg/kg. Following the dosing period, liver was harvested from fed mice, and mRNA levels were determined by RT-PCR. G6PT1, SREBP1 (sterol regulatory element binding factor 1), SREBP2 (sterol regulatory element binding factor 2), ACC1 (acetyl coenzyme A carboxylase alpha), and FAS (fatty acid synthase) mRNA levels were normalized to total RNA in the same samples using 36B4 mRNA. Data are the mean values ± SEM of five mice per treatment group.

**Supplemental Fig. 2.** Liver gluconeogenic enzyme mRNA levels of fed and fasted G6PT1 ASO-treated mice. Total RNA was extracted from livers of fed and fasted (12 h) C57Bl/6 mice (n = 6 per treatment group) treated twice per week (every 3.5 days) with 25 mg/kg control or G6PT1 ASOs for six weeks. G6PT1, G6PC-α, G6PC-β, and PEPCK (phosphoenolpyruvate carboxykinase 1, cytosolic) mRNA levels were determined by RT-PCR. mRNA levels for all genes were normalized to total RNA in the same samples using 36B4 mRNA. Data are the mean values ± SEM of six mice per treatment group.
### Table 1. Metabolic parameters in normal and diabetic mice treated with G6PT1 ASOs.

<table>
<thead>
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<th>ob/ob Mice</th>
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<th>Lean Mice</th>
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<tbody>
<tr>
<td></td>
<td>Fed Control ASO</td>
<td>G6PT1 ASO</td>
<td>Fasted Control ASO</td>
<td>G6PT1 ASO</td>
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<tr>
<td>Body Weight (g)</td>
<td>55.0 ± 2.1</td>
<td>53.5 ± 0.5</td>
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<td>Glucose (mg/dl)</td>
<td>437 ± 65</td>
<td>197 ± 11</td>
<td>214 ± 34</td>
<td>131 ± 16</td>
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<td>Insulin (ng/ml)</td>
<td>11.7 ± 2.1</td>
<td>6.3 ± 1.3</td>
<td>2.4 ± 0.6</td>
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<tr>
<td>Glucagon (pg/ml)</td>
<td>568 ± 125</td>
<td>669 ± 226</td>
<td>404 ± 70</td>
<td>553 ± 59</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>132 ± 9</td>
<td>113 ± 10</td>
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<td>81 ± 14</td>
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<td>Cholesterol (mg/dl)</td>
<td>250 ± 10</td>
<td>251 ± 12</td>
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<td>Liver Triglycerides (mg/g)</td>
<td>49 ± 8</td>
<td>63 ± 4</td>
<td>59 ± 21</td>
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<td>Liver Glycogen (µmol/g)</td>
<td>197 ± 19</td>
<td>238 ± 11</td>
<td>53 ± 9</td>
<td>186 ± 19</td>
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<td>Kidney Glycogen (µmol/g)</td>
<td>7.2 ± 0.27</td>
<td>7.2 ± 0.26</td>
<td>5.2 ± 0.31</td>
<td>6.3 ± 0.47</td>
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All data are from animals that were treated twice per wk (every 3.5 days) by subcutaneous injection with Control ASO 141923 or G6PT1 ASO 149008 for 4-5 weeks. **bold italics**, P<0.05 compared to control ASO at same feeding status.
Table 2. GSD-1b-related parameters in normal and diabetic mice treated with G6PT1 ASOs.

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<th>ob/ob Mice</th>
<th>Lean Mice</th>
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<tr>
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<td>Fed</td>
<td>Fasted</td>
</tr>
<tr>
<td></td>
<td>Control ASO</td>
<td>G6PT1 ASO</td>
</tr>
<tr>
<td>Uric Acid (mg/dl)</td>
<td>0.49 ± 0.07 0.24 ± 0.04</td>
<td>0.39 ± 0.08 0.47 ± 0.08</td>
</tr>
<tr>
<td>Lactate (mg/dl)</td>
<td>116 ± 11 58 ± 4</td>
<td>29 ± 4 64 ± 4</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>275 ± 39 238 ± 60</td>
<td>247 ± 51 236 ± 27</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>216 ± 32 226 ± 53</td>
<td>176 ± 29 241 ± 22</td>
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<tr>
<td>WBC (th/ml)</td>
<td>7.74 ± 1.24 6.38 ± 0.55</td>
<td>5.18 ± 0.9 5.11 ± 1.63</td>
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<tr>
<td>Neutrophils (th/ml)</td>
<td>1.22 ± 0.17 1.1 ± 0.19</td>
<td>0.79 ± 0.15 0.84 ± 0.31</td>
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<tr>
<td>G-CSF (pg/ml)</td>
<td>171 ± 24 169 ± 17</td>
<td>295 ± 88 156 ± 10</td>
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All data are from animals that were treated twice per wk (every 3.5 days) by subcutaneous injection with Control ASO 141923 or G6PT1 ASO 149008 for 4-5 weeks. **bold italics**, P<0.05 compared to control ASO at same feeding status.
Figure 1. Target reduction, decreased G6Pase translocase activity, and glucose-lowering in diabetic mice treated with G6PT1 ASOs.
Figure 2. Hepatic glucose metabolism in mice treated with G6PT1 ASOs.
Figure 3. Liver Histopathology
Figure 4. Hypoglycemic assessment in normal mice treated with G6PT1 ASOs.
Figure 5. Liver and kidney G6PC phosphohydrolase activity and mRNA levels in G6PT1 ASO-treated ob/ob mice
Supplemental Figure 1
Supplemental Figure 2
Specific reduction of hepatic glucose-6-phosphate transporter-1 ameliorates diabetes while avoiding complications of glycogen storage disease
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