Lethal hypoglycemic ketosis and glyceroluria in mice lacking both the mitochondrial and the cytosolic glycerol phosphate dehydrogenases

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Running Title: Combined GPD deficiency
Summary
The activities of either the mitochondrial or cytosolic glycerol phosphate dehydrogenase (mGPD, cGPD) plus that of glycerol kinase are required for the use of glycerol in aerobic metabolism and gluconeogenesis. A knockout mouse lacking mGPD has reduced body weight and fertility, but shows remarkably normal liver and muscle metabolite levels. The BALB/cHeA mouse strain, which lacks cGPD, breeds well and is phenotypically normal, although it demonstrates metabolite abnormalities in certain tissues. Crosses were made between these two strains, and mice were generated that lacked both dehydrogenases. These mice, although active and nursing well for several days, failed to grow, and usually died within the first week. Liver glycerol phosphate levels were elevated 30-fold, while liver ATP, ADP, and AMP levels were reduced by 30-40%. Plasma glycerol was elevated 30 to 50-fold to 30-50 mM, and urine glycerol exceeded 0.45 M (4% wt/vol). GPD-deficient mice were hypoglycemic, had a 50% increase in plasma free fatty acids, and developed ketonuria within the first day of life. Uncoupling protein-1 mRNA in brown adipose tissue was reduced 60%. These mice share some features of both glycerol kinase deficiency and hereditary fructose intolerance, suggesting the phenotype may be due to the combined effects of the loss of a gluconeogenic substrate, the osmotic effects of glycerol, and the metabolic effects of the accumulation of a phosphorylated metabolite.

Introduction
Mammalian cells contain two glycerol phosphate dehydrogenase enzymes. The NADH-dependent cytosolic enzyme (E.C. 1.1.1.8) catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to glycerol phosphate. This reaction is reversible, with a strong preference under physiologic conditions for the production of glycerol phosphate. In the mouse, this enzyme is encoded by Gdc1. An embryonic form of the enzyme is encoded by Gdc2, but has not been found in liver or kidney during gestation, although it persists in brain of the neonate for several weeks (1) and in epididymal white fat for at least 5 days.
after birth (2). The FAD-dependent mitochondrial GPD (E.C. 1.1.99.5) is encoded by a single gene, \textit{Gdm1}, on chromosome 2 (3) and is located on the outer surface of the mitochondrial inner membrane. mGPD catalyzes the irreversible conversion of glycerol phosphate to DHAP, with transfer of electrons from bound FAD through ubiquinone to complex III of the electron transport chain. These two enzymes form the glycerol phosphate shuttle, which cycles glycerol phosphate and DHAP to oxidize NADH formed in the cytosol. A spontaneous mutation in \textit{Gdc1} in the inbred strain BALB/cHeA results in an altered mRNA size and the loss of cGPD enzyme activity (4). cGPD deficient BALB/cHeA animals are viable and fertile, although they demonstrate some evidence of an inhibition of glycolysis at glyceraldehyde phosphate dehydrogenase in skeletal muscle (5). We and others (6) have produced knockout mice deficient in mGPD. The mGPD knockout mice have decreased adiposity and reduced fertility and viability. Alterations are seen in liver metabolites (decreased ATP, increased glycerol phosphate) only in nursing pups.

The glycerol phosphate dehydrogenases also form an important link between carbohydrate and lipid metabolism. DHAP derived from carbohydrates is converted by cGPD to glycerol phosphate, which can be acylated to form phospholipids or triglycerides. An alternate pathway, acylation of DHAP directly by dihydroxyacetone phosphate acyltransferase (7), apparently compensates for the loss of cGPD in mice of the BALB/cHeA strain, which are able to grow and produce normal fat depots, even when placed on a fat free (triglyceride/glycerol free) diet at weaning (5). In tissues that contain glycerol kinase, metabolism of glycerol phosphate by mGPD should allow the conversion of glycerol to DHAP without perturbation of the cytosolic NAD/NADH ratio. DHAP is then available for either the oxidative pathway or gluconeogenesis. Under conditions of glycerol loading, mGPD appears to play a major role in glycerol oxidation (8-10). In the absence of mGPD, glycerol phosphate metabolism is handled solely by cGPD, with remarkably few metabolic abnormalities seen primarily in nursing pups. In the absence of both the cytosolic and mitochondrial enzymes, the link between carbohydrate and fat
metabolism is extremely limited. Triglyceride glycerol can be produced from carbohydrate indirectly through the DHAP-acyltransferase pathway, however, glycerol and glycerol phosphate derived from fat are not able to enter the oxidative or gluconeogenic pathways.

We report here that mice deficient in both GPDs are unable to use glycerol, developing hypoglycemia, ketonuria, glyceroluria, elevated liver glycerol phosphate, profound growth failure, and death within a week of birth.

**Experimental procedures**

_**Animals**_—BALB/cHeA (cGPD null) and C57BL/6J mice were obtained from Jackson Laboratory, Bar Harbor, ME. The mGPD knockouts were constructed as previously described. Briefly, exons 5 and 6 were replaced by a Neomycin marker gene. Targeted RW4 cells (129X1/SvJ origin) were injected into blastocysts derived from the C57BL/6J strain and resulting chimeras were bred to C57BL/6J animals to produce heterozygous animals. Homozygous F2 or F3 mGPD knockout animals were bred with the BALB/cHeA strain to produce animals heterozygous for both the cytosolic and mitochondrial GPD deficiencies. These animals were intercrossed and typed. To simplify further breeding, animals homozygous for the cGPD deficiency and heterozygous for the mGPD knockout (c_0_0/m_+_) were intercrossed to produce animals deficient in both enzymes (c_0_0/m_0_0).

Additional crosses were performed to produce a c_+/+m_+/+ stock.

_Genotyping_—Tail DNA was prepared and mGPD was typed using a three primer PCR. Typing for cGPD status was performed initially by screening for the BALB/c allele of the linked marker D15Mit242, which gives a 104 bp product from both the 129X1/SvJ and C57BL/6J alleles of the mGPD knockout mouse line, and a 90 bp product from the BALB/cHeA allele. Mouse MapPair primers were purchased from Research Genetics, Inc. (Huntsville, Alabama) and used according to methods provided. PCR products were
separated on 3.5% Metaphor agarose (FMC BioProducts, Rockland, ME) according to the manufacturer’s instructions. cGPD status was confirmed in selected cases by enzyme assay on liver homogenates using standard spectrophotometric methods (12).

**Diet**—Mice were routinely fed a standard chow diet (Teklad 8604, 4% fat, Harlan Teklad, Madison, WI). Mice on the C57BL/6J background were maintained on breeder chow (Teklad 8626, 10% fat).

**Expression analysis**—Total RNA was prepared from the interscapular brown adipose tissue using TRI-Reagent® (Molecular Research Center, Inc., Cincinnati, OH) according to directions provided. Real time quantitative reverse transcription polymerase chain reaction was performed using the ABI Prism 7700 sequence detection system and UCP1 primers as described (13).

**Biochemical Assays**—Enzymes and metabolites were measured as previously described (5), except that due to the small size of pups and organs, mouse pups were killed by beheading, and tissues for metabolites (50-100mg) were removed as rapidly as possible and directly homogenized in 6% perchloric acid at 4ºC. Free fatty acids were measured using the Free fatty acids, Half-micro test kit (Roche Molecular Biochemicals, Indianapolis, IN), according to directions. Glucose was measured using a One-Touch Profile instrument (Lifescan, Milpitas, CA). Urine was collected by postmortem aspiration from the bladder using an insulin syringe. Acetoacetic acid was assessed semi-quantitatively on 6-10 µl of urine using Ketostix (Bayer Corporation) according to directions. Pooled urine was used for assay of electrolyte content by the University of Wisconsin Hospitals Clinical Lab, and individual urine samples were assayed for organic acids by gas chromatography/mass spectroscopy (GC/MS) at the University of Wisconsin Biochemical Genetics Laboratory (Madison, WI). Protein assays were performed according to the Lowry method (14) on the sonicated pellets
derived from perchloric acid precipitation. Separate determinations were made of the protein content of whole liver tissue for calculations of molarity.

**Results:**

*Mice lacking both GPDs exhibit growth failure and die prior to weaning*—In a cross between the mGPD knockout strain (c\(^{o/o}\)m\(^{o/o}\)) and the BALB/cHeA strain (c\(^{o/o}\)m\(^{+/+}\)), 0 of 56 F2 animals were deficient in both the cytosolic and mitochondrial GPDs (c\(^{o/o}\)m\(^{o/o}\)) and GPD m\(^{o/o}\) mice were underrepresented (6/56, expected 14/56, p<0.05). In subsequent crosses between animals lacking cGPD and heterozygous for the mGPD knockout (c\(^{o/o}\)m\(^{o/+}\) x c\(^{o/o}\)m\(^{o/+}\)), 0 of 61 weaned animals were mGPD knockouts (expected = 15, p <0.001). Examination of young litters revealed discrepancies in size within a few days of birth (Figure 1A). Runted animals, usually weighing less than 2 grams at 5 days of age (vs. a normal weight of 3.5 g), were confirmed to be homozygous for the mGPD deletion and lacking cGPD activity. These animals were noted to have little subcutaneous fat, although most were active and well fed for several days. By 5-7 days of age most of these GPD deficient animals stopped feeding and were lethargic. Fig. 1A may be misleading with regard to weight gain, as most of the GPD deficient animals died within 2-7 days of birth, and those surviving longer tended to be larger than average. Similar results were found in c\(^{o/o}\)m\(^{o/o}\) pups derived from c\(^{o/+}\)m\(^{o/o}\) intercrosses (data not shown). Analysis of pups within the first four days showed that mice deficient in both GPDs were born in expected numbers (23/86) and were of normal size at birth. Weights on day 1 did not differ significantly: the mean weight ± standard deviation of c\(^{o/o}\)m\(^{o/o}\) pups was 1.39 ± 0.11 g (N=9), and that of c\(^{o/o}\)m\(^{o/+}\), 1.43 ± 0.18 g (N=16).

*Histology*—Consistent with the gross appearance of the GPD deficient pups, histology showed little, if any, subcutaneous fat in 5-7 day old c\(^{o/o}\)m\(^{o/o}\) animals. Brown adipose tissue
appeared normal. Other tissues showed no difference from healthy siblings, with the exception of decreased extramedullary hematopoiesis in the livers of the $c^{o/o} m^{o/o}$ animals.

**GPD deficient animals are hypoglycemic**—Blood glucose values rose with age and weight in all pups tested, except those lacking both GPDs (Fig. 1 B,C). Data from $c^{+/+} m^{+/+}$ pups did not differ from $c^{o/o} m^{+/+}$ and $c^{o/o} m^{+/+}$ animals, and are not shown. There were several sporadic high blood glucose values in the $c^{o/o} m^{o/o}$ pups, but most had low glucose values that remained near neonatal levels. Some variation in glucose values may be expected, as we did not attempt to control for feeding.

**GPD deficient animals are ketotic**—Urine acetoacetate was positive in all $c^{o/o} m^{o/o}$ pups tested (N=19), and ranged from trace-small (5-15 mg/dl) to moderate-large (40-80 mg/dl). Ketones were negative in all other pups tested, including 29 cGPD null, 5 mGPD knockout, 4 wild type (BALB x C57BL/6J) controls, and 3 C57BL/6J controls. Both acetoacetate and 3-hydroxybutyrate were identified by GC/MS in urine samples from two $c^{o/o} m^{o/o}$ animals, but not in urine from two control animals ($c^{o/o} m^{+/+}, c^{o/o} m^{+/+}$).

**GPD deficient animals have low liver adenylates and elevated glycerol phosphate and free fatty acids**—ATP values were reduced 40% in the $c^{o/o} m^{o/o}$ pups vs. mGPD positive littermates, and ADP and AMP values were also proportionately reduced, with total adenylates reduced by 31-39% (Table 1). ATP values were increased in cGPD null pups ($c^{o/o} m^{+/+}$) vs. wild controls ($c^{+/+} m^{+/+}$). Liver glycerol phosphate values were doubled in $c^{o/o} m^{+/+}$ vs. $c^{o/o} m^{o/o}$, and were elevated 16-32 fold in $c^{o/o} m^{o/o}$ littermates. In spite of the elevated liver glycerol phosphate, plasma free fatty acids were slightly elevated in the GPD deficient animals, although the range of free fatty acid values in the GPD deficient animals (0.38-1.35mM) slightly exceeded that of littermate controls (0.4-1.0 mM) in both high and low values.
**GPD deficient animals develop an elevated plasma glycerol and lose glycerol in their urine**— Plasma and urine glycerol were elevated in all pups lacking both GPDs, and these values increased with age (Figure 2). Values of plasma glycerol in normal animals averaged 0.8 mM, ranged from 0.2-1.3 mM, and did not change appreciably with age, whereas values in c^{o/o} m^{o/o} pups ranged from 10.8-74 mM (Fig. 2A). These values are similar to those reported in the glycerol kinase knockout mouse, which also has growth failure and dies in 3-4 days (15). In urine, no glycerol value in the c^{o/o} m^{+/+} controls exceeded 0.4 mM, while values in the c^{o/o} m^{o/o} pups ranged from a low of 57 mM on day 1, to a high of 478 mM (4% glycerol) on day 4 (Fig. 2B). On GC/MS, glycerol was the most prominent peak in urine from c^{o/o} m^{o/o} animals, but a minor peak in controls (data not shown). While elevated urine glycolate was reported in glycerol kinase knockout animals (15), no glycolate was detected in our samples.

**GPD deficient animals have low UCP1 mRNA levels**—mGPD has been proposed to play a role in thermogenesis (16,17), however we were unable to find a significant alteration in thermogenesis in mGPD knockout animals, which have normal UCP1 mRNA levels^2. We evaluated UCP1 mRNA in brown adipose tissue of 3-5 day old pups from c^{o/o} m^{+/+} intercrosses. Pups lacking both GPDs had UCP1 mRNA levels that were reduced 60% compared with littermate controls. UCP1 mRNA levels (arbitrary units) ± S.E., (N) were as follows: GPD c^{o/o} m^{+/+}, 6698± 372 (4); c^{o/o} m^{o/+}, 6188± 433 (13); c^{o/o} m^{o/o}, 2483 ± 335 (8). † p<.01, ‡ p<.001 vs. c^{o/o} m^{o/o}. This reduction may reflect the general poor condition of the GPD deficient animals, or the hormonal consequences of hypoglycemia. Chronic hypoglycemia would be expected to elevate corticosterone, which has been shown to inhibit UCP1 transcription (18,19). Low UCP1 levels could impair the ability of the GPD deficient pups to maintain body temperature.
Urinalysis and hematology—Table 2 shows the results of urine electrolytes performed on pooled urine from 1-3 day old c/o m/c pups and two groups of controls. Results are consistent with sodium loss due to ketoacidosis, an osmotic diuresis, or proximal tubular dysfunction. Analysis of these data is limited by the lack of information on milk intake, urine output, and other electrolyte losses. The electrolyte composition of mouse milk itself is uncertain, as very different values have been reported using microelectrode methods (Na $^+$ 76.9, K $^+$ 32.5, Cl $^-$ 41.6) (20) and atomic absorption methods (Na $^+$ 23.5, K $^+$ 50.4) (21). The GPD deficient animals were also found to have elevated plasma proteins (114 ± 3% of littermate controls, N=11 controls, 7 GPD-deficient, p<0.02) and a tendency toward elevated hematocrits (109%, N=3 each), consistent with the presence of severe dehydration in these animals.

Discussion:
Gluconeogenesis and the importance of glycerol—Glyceride glycerol is an especially important gluconeogenic precursor in the neonatal mouse, as 80% of calories from mouse milk are derived from fat, 16-17% from protein, and only 2-5% from lactose (20, 22-24). Thus total calories available from dietary glycerol (approximately 4%) equal calories from lactose. As fatty acids cannot be converted to glucose and dietary protein is needed for growth, glyceride glycerol could provide 15-25% of glucose production in the fed state, and possibly more when fasting.

In GPD-deficient pups, liver glycerol kinase produces glycerol phosphate, which cannot be metabolized to dihydroxyacetone phosphate. Other substrates are therefore required for gluconeogenesis, however gluconeogenesis from glycerol is energetically less expensive than that from amino acids and citric acid cycle intermediates. For example, production of one glucose molecule from pyruvate consumes 4 ATP, 2GTP, and 2 mitochondrial NADH. Oxidation of each NADH could otherwise produce 3 ATPs, so the net ATP loss is 12 ATP per glucose molecule created. Synthesis of one glucose molecule from glycerol requires 2
ATP and produces either 2 FADH$_2$ or 2 cytosolic NADH (equivalent to 2-3 ATP each), for a net gain of 2-4 ATP. In a rapidly growing animal, increased gluconeogenesis from amino acids and citric acid cycle intermediates may therefore deplete both the substrates and the energy required for growth (Figure 3).

**Mechanism and consequences of ketogenesis**—Control of ketogenesis occurs primarily through the inhibition of carnitine palmitoyl transferase I (CPTI) by malonyl CoA (25) and the inhibition of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA synthase) by succinyl-CoA-dependent auto-succinylation (26,27). In the presence of an elevated glucagon/insulin ratio, both inhibitions are released. The neonatal rodent, with its low sugar and high fat diet, has elevated glucagon and low levels of insulin in order to maintain gluconeogenesis and fatty acid oxidation (28). In the combined GPD deficiency, loss of a major gluconeogenic substrate might be expected to exaggerate this response (Figure 3). In addition, low levels of adenylates and depletion of P$_i$ could limit coupled respiration, slowing fatty acid oxidation in the liver of the GPD-deficient pups, and therefore increasing the proportion of acetyl-CoA used for the production of ketones (Figure 3). The loss of ketones in the urine is coupled with an obligatory loss of cations in the urine, generally Na$^+$, K$^+$, and NH$_4^+$. These losses can lead to dehydration, electrolyte disturbances, and excessive protein consumption.

**Effect of glycerol phosphate accumulation**—In the absence of both GPDs, there is apparently no mechanism to prevent the accumulation of glycerol phosphate in tissues that contain glycerol kinase. In liver and kidney, glycerol phosphate is therefore produced, but cannot be converted into dihydroxyacetone phosphate and cannot be used for gluconeogenesis or aerobic metabolism. Although this glycerol phosphate could be used for lipid synthesis, lipid does not accumulate in the liver of GPD deficient mice, most likely due to metabolic conditions favoring fatty acid degradation. It is not clear what limits the
accumulation of glycerol phosphate, however glycerol phosphate is known to be a potent product inhibitor of glycerol kinase (29). Glycerol phosphate levels found in the liver of GPD deficient animals (Table 1) correspond to concentrations of approximately 3-5 mM (calculated from the protein content of mouse pup liver of 160 mg/gm of wet weight). Plasma glycerol increases up to 74 mM (normal 0.2-1.3 mM), while urine glycerol eventually exceeds 400 mM (normal ≤ 0.4 mM). Urine glycerol is almost completely reabsorbed by the kidney up to plasma levels of 1 mM (30). Above this level the urine glycerol concentration depends on the plasma glycerol concentration and the degree of water reabsorption from the urine. High urine glycerol levels could lead to an osmotic diuresis, salt loss, and dehydration, consistent with our findings.

Accumulation of liver glycerol phosphate may at least partially explain the low ATP and total adenylates seen in the GPD-deficient animals. In the rat, an intraperitoneal bolus of glycerol (9) or liver perfusion with ethanol (31) lead to the accumulation of liver glycerol phosphate to 4-14 mM. This glycerol phosphate increase is accompanied by a fall in free Pi (9,31), and a fall in liver ATP and total adenylates. The loss of adenylates is due to activation of AMP deaminase by a fall in Pi levels, and it is accompanied by a rise in the production of breakdown products of adenylate (31). A chronic accumulation of phosphorylated metabolites may be more deleterious. In hereditary fructose intolerance, the accumulation of fructose-1-phosphate also leads to a fall in ATP and Pi in liver and kidney. Adenylate degradation leads to the release of Mg2+ into the blood, and the elevation of serum urate. In affected individuals, severe or prolonged exposure to fructose can lead to renal proximal tubular dysfunction or renal failure, and to liver dysfunction, fibrosis, or liver failure. Even with minimal fructose exposure, stunted growth is common (32). These findings are consistent with the results in our GPD deficient animals, however, low ATP levels are also seen in the livers of mGPD knockout pups, without a large increase in glycerol phosphate. Additional factors may be involved, such as an increased ATP consumption in gluconeogenesis from amino acids.
Elevated glycerol phosphate may also be directly linked to the hypoglycemia observed in the GPD deficient pups. Boluses of glycerol or glycerol phosphate interfere with gluconeogenesis from alanine, especially in malnourished animals (33). The mechanism is not clear. Of the gluconeogenic enzymes, fructose-1,6-bisphosphatase is the most susceptible to inhibition by glycerol phosphate, with an $I_{50}$ of 20mM in the rat (34) and 60 mM in the Chinese hamster (35). At the level of glycerol phosphate seen in our animals, a maximum inhibition of 10-30% might be expected for this enzyme. Gluconeogenesis may also be affected by the low ATP levels in the face of the increased ATP demand for gluconeogenesis from amino acids.

*Comparison of the GPD deficient mouse and the glycerol kinase null mouse*—Recently a glycerol kinase knockout mouse was reported (15) that shows a number of similarities to the GPD deficient mouse. The glycerol kinase deficient mouse has a normal birth weight, but fails to gain weight, has plasma glycerol levels of approximately 40 mM, elevated free fatty acid levels, and dies by 3-4 days of age. Unlike the GPD deficient mice, the glycerol kinase knockout mice are not ketotic, and have normal blood glucose. These differences could be attributed to the inability of the glycerol kinase null mouse to accumulate glycerol phosphate, however, hypoglycemia and episodic ketoacidosis have been reported in patients with isolated glycerol kinase deficiency, although the human diet, including human milk, contains a higher percentage of both sugars and protein than does mouse milk. The lack of hypoglycemia and ketosis in the glycerol kinase knockout is likely attributable to the autonomous glucocorticoid secretion seen in this mouse, which should increase protein catabolism and provide the amino acid substrate needed for adequate gluconeogenesis (albeit at the expense of muscle and other tissues). Glucocorticoid treatment has been used to treat hypoglycemic ketosis of childhood (36) and bovine ketosis (37), two conditions in which a paucity of gluconeogenic substrates has been postulated as the cause of symptomatic hypoglycemia and ketoacidosis. Autonomous glucocorticoid secretion has not
been reported in human glycerol kinase deficiency, and caution should therefore be used in interpretation of the findings in the glycerol kinase null mouse.

**Conclusions**—Mice deficient in both the cytosolic and mitochondrial GPDs usually die within the first week of life. Hypoglycemia, ketonuria, and alterations in liver glycerol phosphate levels and ATP occur during a time period in which the mice are still active and well-fed (as evidenced by stomachs full of milk), and likely all contribute to the eventual decompensation. Death may result from a combination of hypothermia, ketoacidosis and resultant electrolyte disturbances, protein deficiency due to the obligatory use of amino acids for gluconeogenesis, glycerol-induced osmotic diuresis and dehydration, and organ failure secondary to ATP and P$_i$ depletion.

While glycerol kinase and the GPDs are essential for normal glycerol metabolism, compensatory genetic factors clearly play a role in the phenotype resulting from their loss. In the combined GPD deficient mice death occurs within 1-2 days on an inbred BALB/cHeA background (R.A.K., unpublished data), while pups often survive 5-7 days on the genetically mixed background. In man, the phenotype of individuals with isolated glycerol kinase deficiency ranges from episodic ketoacidosis, hypoglycemia, and seizures to completely asymptomatic. This spectrum can be seen within a single family, and is therefore likely affected by other genetic or environmental factors. (38,39).

Studies of mice lacking both GPDs, on various genetic backgrounds, could help to define the compensatory mechanisms responsible for the maintenance of normoglycemia.

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**Footnotes:**

1 The abbreviations used in this paper are: DHAP, dihydroxyacetone phosphate; FAD, flavine-adenine dinucleotide; GC/MS, gas chromatography/mass spectroscopy; (c- or m)GPD, (cytosolic or mitochondrial) glycerol phosphate dehydrogenase; P, inorganic phosphate; UCP1, uncoupling protein-1.

2 Brown, L.J., Koza, R.A., Everett, C., Reitman, M.L., Marshall, L., Fahien, L.A., Kozak, L.P., and MacDonald, M.J. Normal thyroid thermogenesis, but reduced viability and adiposity in mice lacking the mitochondrial glycerol phosphate dehydrogenase. (submitted)
Figure Legends:

Figure 1. Growth failure and hypoglycemia in GPD-deficient mice. A. Weight gain in GPD c/o/c/o pups (black triangles, N=4-9 animals per point) is compared with c/o/c/o and c/o/c/+ littermates (open circles, N=5-23 animals per point). Means ± S.E. are shown. Comparisons were performed by the Student’s t test. *p<.05  ** p<.001  B. GPD-deficient mice showed only a limited increase in blood glucose with age. Symbols as in (A). N=6-18 animals per point, except N=3 for the 5 day c/o/c/o. C. Weights of individual animals are shown to demonstrate the correlation between glucose and weight in control littermates (R²=0.77), and the lack of correlation in the GPD-deficient pups (R²=0.02), in which the glucose generally remained below 60 mg/dl. Symbols are as in (A).

Figure 2. Urine and plasma glycerol. GPD c/o/c/o pups are show by solid triangles, while mGPD positive littermates (c/o/c/+ and c/o/c/+/+ ) are shown by open circles. Urine glycerol in GPD deficient pups ranged from 57-478 mM, while values in all other mice tested were less than 0.4 mM, and did not increase with age. Plasma glycerol values in the c/o/c/o animals ranged from 10.8-74 mM, while those of the c/o/c/+ and c/o/c/+/+ littermates were 0.2-1.3 mM.

Figure 3. Proposed metabolic alterations in combined GPD deficiency. 1. Glycerol phosphate levels rise due to the loss of the GPDs. Phosphate derived from ATP is trapped as glycerol phosphate (G3P), and glucose production is decreased. Glycerol levels rise along with G3P levels, and glycerol is excreted in the urine. 2. P_i is depleted, resulting in degradation of adenylates. 3. Low blood glucose levels result in increased consumption of amino acids for gluconeogenesis. 4. Oxaloacetate (OAA) and malate are diverted from the citric acid cycle towards gluconeogenesis, depleting cycle intermediates while increasing ATP demands. 5. Reductions in ADP and P_i slow oxidative phosphorylation, resulting in
elevated mitochondrial NADH. 6. Low insulin and high glucocorticoid and epinephrine levels result in hydrolysis and decreased synthesis of triglycerides (TG), increasing free fatty acids (FFA). 7. Low insulin and high glucagon levels lead to inhibition of acetyl CoA-carboxylase, releasing the malonyl-CoA inhibition of carnitine palmitoyl transferase I, and accelerating fatty acid degradation to acetyl CoA. 8. Elevated acetyl-CoA, combined with decreased flux through the citric acid cycle, increases production of acetoacetyl-CoA. Lowering of the succinyl-CoA concentration relieves inhibition of the mitochondrial HMG-CoA synthase, increasing the production of acetoacetate and 3-hydroxybutyrate (3-OHB). Loss of these compounds in the urine increases loss of $\text{NH}_4^+$, $\text{Na}^+$, and $\text{K}^+$. 
Table 1

Liver metabolites and plasma free fatty acids

Liver metabolites are shown as nmol/mg protein, and free fatty acids (FFA) as mmol/L. Means are shown ± standard error and the number of samples tested is given in parentheses. Two sets of experiments are shown, the second set was performed later and represents a somewhat more inbred group of animals. Animals were 2-7 days old, and were littermates from intercrosses of GPD c^{0/0} m^{0/+} F_{2} animals, with the exception of animals of the genotype GPD c^{+/+} m^{+/+}, which were maintained as a separate breeding stock derived from the same initial cross. G3P, glycerol phosphate.

| GPD      | ATP       | ADP       | AMP       | G3P       | FFA       |
|----------|-----------|-----------|-----------|-----------|-----------|
| c^{0/0} m^{+/+} | 10.7 ± 1.6 (5)* | 6.5, 7.7 (2) | 4.1, 1.9 (2) | 0.7 ± 0.1 (9) ‡ | ---       |
| c^{0/0} m^{0/0} | 6.0 ± 0.4 (7) | 4.9 ± 0.6 (4) | 1.9 ± 0.5 (4) | 22.9 ± 1.9 (9) | ---       |
| c^{0/0} m^{+/-} | 11.0 ± 0.3 (9) ‡ | --- | --- | 1.9 ± 0.2 (9) ‡ | 0.61 ± 0.05 (6) |
| c^{0/0} m^{+-} | 10.6 ± 0.4 (11) † | --- | --- | 3.7 ± 0.5 (11) ‡ | 0.66 ± 0.07 (11)* |
| c^{0/0} m^{0/0} | 6.6 ± 0.2 (11) † | --- | --- | 31.0 ± 3.3 (11) | 0.91 ± 0.09 (15) |
| c^{+/+} m^{+/+} | 8.8 ± 0.2 (5) † † | --- | --- | 1.3 ± 0.09 (5) ‡ †† | 0.38 ± 0.06 (3) |

Pair wise comparisons were performed using the Student’s t-test. *p ≤ .05 vs. c^{0/0} m^{0/0}, † p ≤ .01 vs. c^{0/0} m^{0/0}, ‡ p ≤ .001 vs. c^{0/0} m^{0/0}. **p ≤ .05 vs. c^{0/0} m^{+/+}, †† p ≤ .01 vs. c^{0/0} m^{+/+}. **
Table 2

*Urine electrolytes*

Urine was collected and stored at –20º C. Samples were pooled after animals were genotyped. Number of samples pooled (age): 10 c<sup>o/o</sup>m<sup>o/o</sup> (1-3 days), 8 c<sup>o/o</sup>m<sup>(o or +)/+</sup> (1-3 days), 2 c<sup>o/o</sup>m<sup>+/+</sup> (6 days). Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup> are in mmol/L, osmolarity is in mOsm/L.

| Genotype       | Age   | Na<sup>+</sup> | K<sup>+</sup> | Cl<sup>-</sup> | Osm  |
|----------------|-------|---------------|---------------|---------------|------|
| GPD c<sup>o/o</sup>m<sup>o/o</sup> | 1-3 days | 44            | 53            | 75            | 485  |
| GPD c<sup>+/+</sup>m<sup>(o or +)/+</sup> | 1-3 days | 15            | 102           | 132           | 416  |
| GPD c<sup>o/o</sup>m<sup>(o or +)/+</sup> | 6 days     | 8             | 59            | 41            | 369  |
Figure 1
FIGURE 2

A. PLASMA GLYCEROL

B. URINE GLYCEROL
Lethal hypoglycemic ketosis and glyceroluria in mice lacking both the mitochondrial and the cytosolic glycerol phosphate dehydrogenases
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